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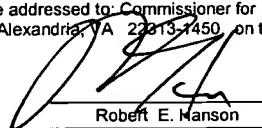
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October 24, 2003

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Mail Stop Appeal Brief-Patents

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Re: SN 09/698,789 "METHOD FOR PLANT BREEDING" – Michael Spencer, et al.
Our Ref. DEKM:157USC1; Client Ref. [38-15(51223)];

Commissioner:

Transmitted herewith for filing are:

1. An Appeal Brief (an original and two copies), including a Petition for Extension of Time;
2. A check for \$440.00 (\$330 to cover the appeal brief filing fee and \$110 for the one-month extension of time); and
3. A return postcard to acknowledge receipt of these materials. Please date stamp and mail this postcard.

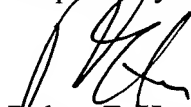
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Respectfully submitted,



Robert E. Hanson
Reg. No. 42,628

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PATENT

UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Michael Spencer, Rita Mumm,
J. Jefferson Gwynn, David McElroy and
Michael A. Stephens

Serial No.: 09/698,789

Filed: October 27, 2000

For: METHOD FOR PLANT BREEDING (AS
AMENDED)

Group Art Unit: 1638

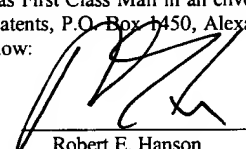
Examiner: Kruse, David H.

Atty. Dkt. No.: DEKM:157USC1

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BRIEF ON APPEAL

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APPENDIX 1: Appealed Claims

APPENDIX 2: Exhibits

Exhibit A — Kyojuka *et al.* (*Plant Cell*, June; (6): 799–810, 1994)

Exhibit B — Hamilton *et al.*, (*Plant Mol Biol.* Jan; 18(2):211-8, 1992)

Exhibit C — Declaration of Dr. Paul C.C. Feng

PATENT

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents

Commissioner for Patents

P.O. Box 1450

Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief in response to the Final Office Action dated April 22, 2003. This Brief is filed pursuant to the Notice of Appeal mailed July 21, 2003. The date for filing the instant Brief is October 24, 2003, based on the receipt of the Notice of Appeal by the Patent and Trademark Office on July 24, 2003 and Petition for Extension of Time of one-month enclosed herewith. The fees for filing this Appeal Brief and the extension of time are enclosed. Please date stamp and return the attached postcard as evidence of receipt.

PETITION FOR EXTENSION OF TIME

Pursuant to 37 C.F.R. § 1.136(a), Appellants petitions for an extension of time of one month to and including October 24, 2003, in which to file this Appeal Brief. Pursuant to 37 C.F.R. § 1.17, a check in the amount of \$440.00 is enclosed, which includes the process fee (\$110.00) for a one-month extension of time. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski Deposit Account No. 50-1212/DEKM:157USC1.

I. REAL PARTIES IN INTEREST

The real party in interest is Monsanto Company, the parent company of assignee DeKalb Genetics Corp.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-118 were originally filed. Claims 1-82, 88-95, and 100-118 were deleted in a Preliminary Amendment filed concurrently with the application on October 19, 2000. No other claims have been deleted or added. Claims 83-87 and 96-99 were therefore pending at the time of the Final Office Action. The final rejection of claims 83-87 and 96-99 is the subject of the instant appeal. A copy of the appealed claims is attached hereto as Appendix 1.

IV. STATUS OF AMENDMENTS

No claim amendments have been made subsequent to the Final Office Action.

V. SUMMARY OF THE INVENTION

The invention relates to a method of plant breeding. Specification at page 73, lines 24-27. More particularly, it concerns methods of plant breeding with maize plants comprising an EPSPS transgene that renders the plant vegetatively and female reproductively tolerant to the herbicide glyphosate, but not male reproductively tolerant so that glyphosate treatment renders the plant male sterile. Specification at page 74, lines 26-28. Following application of glyphosate, the treated plants are male sterile but remain female reproductively viable. The plants are then

capable of being pollinated by a second plant to produce hybrid progeny without the need for removal of male reproductive parts by detasseling, which is the standard method of maize hybrid production. Specification at page 75, lines 10-14.

VI. ISSUES ON APPEAL

(A) Are claims 83-87 and 96-99 properly rejected under 35 U.S.C. § 112, first paragraph, as lacking an adequate written description in the specification?

(B) Are claims 83-87 and 96-99 properly rejected under 35 U.S.C. § 112, first paragraph, as not being enabled by the specification?

VII. GROUPING OF THE CLAIMS

The claims stand or fall together for purposes of this appeal.

VIII. SUMMARY OF THE ARGUMENT

The written description rejection was improperly maintained. The claims are directed to a method of plant breeding. The steps of the claimed method employ maize plants comprising an EPSPS transgene conferring resistance to the herbicide glyphosate in vegetative tissues but wherein the plants are rendered male sterile by treatment with glyphosate. As described in the specification, this characteristic results from the minimal expression of the EPSPS transgene in the male reproductive tissues. Appellants illustrate the invention with two working examples of independent transformation events that exhibit glyphosate-inducible male sterility. The fact that independent transformation events with the characteristic were obtained demonstrates possession of the glyphosate-inducible male sterile plants and repeatability of the characteristic. This was confirmed by the Declaration of Dr. Paul C.C. Feng. Further, the transformation events

described in the specification can be transferred into any maize genotype. This is also illustrated in the working examples. The teaching in the specification allows production of numerous other glyphosate-inducible male sterile plants, as shown in the Declaration of Dr. Feng. This is underscored by the advanced state of the art for tissue-specific expression of transgenes in plants at the time the application was filed. The claimed invention has therefore been fully described in the specification in compliance with the written description requirement and the Examiner has provided no basis to doubt this description. Reversal of the rejection is thus respectfully requested.

The enablement rejection was also improperly maintained. The working examples, description in the specification and Declaration of Dr. Feng demonstrate the enablement of the full scope of the claims. The Examiner has even acknowledged that enablement has been provided for use of promoters from three diverse sources. The Examiner has provided no basis to doubt why the acknowledged examples alone are not representative of the full scope of the claims. The Declaration of Dr. Feng further establishes enablement for numerous other promoters. Again, this is underscored by the advanced state of the art regarding tissue-specific expression of transgenes. The Examiner has disregarded Appellants' objective evidence without providing a basis for doing so. This contravenes the requirement of the APA that substantial evidence be provided in support of any rejection. Reversal of the rejection is thus requested.

IX. ARGUMENT

A. The Written Description Rejection Was Improperly Maintained

The Examiner finally rejected claims 83-87 and 96-99 under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to convey to one of skill in the art that Appellants were in possession of the

claimed subject matter. In particular, it was alleged that Appellants were not in possession of glyphosate-inducible male sterile plants other than events GG25 and GJ11.

(1) Appellants have demonstrated possession of the invention

The invention relates to the finding that maize plants may be prepared expressing an EPSPS transgene conferring resistance to the herbicide glyphosate (*e.g.*, Roundup®) wherein the plants are rendered male sterile by treatment with glyphosate. For example, pages 78 and 79 of the specification explain that expression of glyphosate-resistant EPSPS in vegetative tissues, with little or no expression in male reproductive parts (*e.g.*, pollen), yields plants that are rendered male-sterile by glyphosate treatment but remain viable and female fertile.

This concept was confirmed by studies set forth in the Feng Declaration submitted with Appellants' Response to the First Office Action. **[Exhibit C]**. As described by Dr. Feng, histological studies of maize treated with glyphosate demonstrated that glyphosate arrests the maturation of microspore pollen cells, resulting in inviable pollen and male sterility. The studies indicated that the impact of glyphosate was focused during the development of pollen; specifically during the development of the microspore mother cell, tetrad, and microspores. **[Exhibit C, para. 5-6]**.

The studies also demonstrated that pollen with little or no expression of a glyphosate-resistant EPSPS transgene is susceptible to glyphosate, whereas pollen expressing high levels of resistant EPSPS is not. In particular, immunolocalization studies were carried out on male fertile glyphosate-resistant plants as well as plants exhibiting glyphosate-inducible male sterility **[Exhibit C, para. 6]**. Plants that remained male fertile after glyphosate treatment showed high expression of the glyphosate-resistant EPSPS in the tapetum, microspore mother cell, tetrad and microspores. In contrast, plants that exhibited vegetative glyphosate tolerance but male

reproductive intolerance (glyphosate-inducible male sterility) displayed low to no expression in the same tissues. The results confirmed that minimal expression of the glyphosate-resistance transgene relative to vegetative tissues yields a plant that is vegetatively tolerant to glyphosate but is inducibly male sterile. Dr. Feng's results therefore confirmed the general applicability of the invention and that Appellants were in possession of the invention.

Dr. Feng concluded, based on the studies and teaching in the specification, that one of skill in the art in possession of the application could “*readily prepare transgenic plants with a glyphosate-inducible male sterile phenotype using many different combinations of promoters and glyphosate resistant EPSPS transgenes without undue experimentation.*” (emphasis added, **Exhibit C**, para. 9). This demonstrates possession of the invention by Appellants. The Examiner has done nothing to counter this showing. Instead, the Examiner merely disregards the evidence of Appellants and replaces Dr. Feng's opinion with the Examiner's own. This is improper and the rejection must therefore be reversed.

(2) The working examples describe the full scope of the claims

Appellants demonstrated the function of the invention using transformation events GG25 and GJ11, but the invention is in no way limited to these events. These events are illustrative of the full scope of the invention in that they demonstrate the broadly applicable characteristic of glyphosate-inducible male sterility. The Examiner has provided no basis to conclude otherwise. The Examiner has acknowledged that the application teaches maize plants comprising an EPSPS transgene operably linked to a CaMV35S promoter, maize histone promoter or *Arabidopsis* histone promoter. Again, all that is significant is that the EPSPS is not highly expressed in pollen so that resistance is not present.

Further, plant breeding techniques that are well known in the art and described in the application allow the transfer of a transformation event conferring glyphosate-inducible male sterility into essentially any maize genotype. Therefore, even a single transformation event would allow use of plants of any genotype. This, as well as procedures for introgression of a transgene into different maize plants and plants made thereby are fully described in the specification. For instance, Example 15 at pages 94-96 of the specification describes marker assisted breeding of EPSPS transgenes for such introgression. In Example 14 at pages 92-93, the specification describes the introgression of the GG25 and GJ11 transformation events into elite inbreds and hybrids of maize. In these studies, the GG25 and GJ11 transformation events were each introgressed by backcrossing into the elite inbred lines FBLL (U.S. Patent Appl. No. 08/181,708, filed January 14, 1994) and NL054B (U.S. Patent Appl. No. 08/595,549, filed February 6, 1996). Numerous different plants containing the transgenes were produced over each backcross generation.

Two exemplary hybrids that contained these events were also produced, designated DK626 and DK580. DK580 hybrids were produced by a cross of FBLL to MBZA (U.S. Pat. Appl. No. 08/182,616, filed January 14, 1994) and DK626 hybrids were produced by a cross of NL054B by MM402A (U.S. Pat. Appl. No. 08/181,019, filed January 13, 1994), thereby yielding hybrids containing the respective transformation events. The hybrids were field tested for yield and other agronomic characteristics as well as herbicide tolerance. In Example 12, at pages 90-91 of the specification, it was shown that the hybrids produced exhibited male sterility upon application of glyphosate at the V8 stage of glyphosate application (FIG. 8B), but not during earlier stages of development, when pollen was less fully formed; *e.g.*, at the V4 stage of

application (FIG. 8A). Similar procedures could be carried out with other transgenes for introduction into any genotype. The invention has therefore been fully described

(3) The steps of the claimed method have been fully described

With regard to the breeding steps set forth in claim 83, these are described, for example, at pages 73-77 of the specification. Described are specific examples of breeding protocols within the scope of the claims. In addition to methods for the production of hybrid corn seed using inducible male-sterility, types of inbred parent lines are also described, such as male and female parents that are elite and derived from different heterotic backgrounds, and into which one or more appropriate transformation events have been backcrossed.

Techniques for fertilization are also described in the specification, including natural or mechanical techniques. Natural pollination occurs in corn when wind blows pollen from the tassels to the silks that protrude from the tops of the incipient ears, whereas artificially directed pollination can be effected either by controlling the types of pollen that can blow onto the silks or by pollinating by hand. Further given are examples of different developmental stages for glyphosate treatments. Still further provided are descriptions of how rows are planted and harvested. Techniques for applying herbicides, including glyphosate, are also given at pages 20-25 of the specification.

(4) Written description must be analyzed based on the claimed invention and in view of the level of skill in the art

Appellants finally note that the claims are directed to a method of plant breeding comprising the recited steps. The steps involve the use of a plant that exhibits glyphosate-inducible male sterility, but Appellants are not claiming the plant itself. The relevant written description inquiry here is thus not whether written description has been established for claims to

glyphosate-inducible male sterile plant, but rather whether the claimed method of plant breeding has been adequately described to show possession of the invention. The Examiner nonetheless appears to have examined the claims as if they were composition claims directed to the glyphosate-inducible male sterile plants.

While a glyphosate-inducible male sterile plant must be used to practice the invention, Appellants need not describe this plant in the same manner as if claimed in a composition claim. The focus of the written description inquiry must be on the steps recited in the claims. This is because what is required to meet the written description requirement is that the applicant show that he or she was in possession of the *claimed invention*. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991). To meet this standard, it is only required that the specification allow persons of ordinary skill in the art to recognize that the applicants invented *what is claimed*. *In re Gosteli*, 872 F.2d 1008, 1012 (Fed. Cir. 1989) (citations omitted). The full scope of the claimed method has been described as set forth herein.

Written description must also be reviewed from the perspective of one of skill in the art at the time the application is filed. *Wang Labs., Inc. v. Toshiba Corp.*, 993 F.2d 858, 863 (Fed. Cir. 1993). The Examiner has failed to consider that the tissue-specific expression of transgenes in plants was well known at the time of filing the application. This must be taken into account.

Numerous published references establish the detailed knowledge in the art of tissue-specific expression of transgenes in plants. For example, Kyojuka *et al.* (*Plant Cell*, June; (6): 799–810, 1994) describe delineation within the maize alcohol dehydrogenase 1 (Adh1) promoter of sequences that confer tissue-specific expression. Regions responsible for various different types of tissue specific expression were identified using expression of marker genes. [Exhibit A, Abstract]. Pollen-specific expression in particular was found to require a sequence outside the

promoter region, between +54 and +106 of the untranslated leader, as well as a silencer element in the promoter between -72 and -43. Similarly, Hamilton *et al.* (*Plant Mol Biol.* Jan; 18(2):211-8, 1992) describe the identification of sequences in a region from -100 to -54 responsible for pollen-specific expression by the promoter of the Zm13 gene from *Zea mays*. [Exhibit B, Abstract].

These and many other references from prior to the filing of the application demonstrate that tissue-specific expression of transgenes was well known. These show that even that the particular sequences responsible for tissue specific expression within a given promoter could be identified and manipulated. This information further demonstrates the written description of the claims and must be considered by the Examiner. *See, e.g., In re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d 1527, 1534-35 (Fed. Cir. 1992).

The substantial evidence presented herein above more than adequately demonstrates full compliance with the written description requirement. Reversal of the rejection is thus respectfully requested.

B. The Enablement Rejection Was Improperly Maintained

The Examiner finally rejected claims 83-87 and 96-99 under 35 U.S.C. 112, first paragraph, as not being enabled by the specification. The Examiner acknowledges that the claims are enabled for plant breeding using a female maize parent plant comprising an EPSPS transgene operably linked to a CaMV35S promoter, maize histone promoter or *Arabidopsis* histone promoter. However, the Examiner alleges that enablement has not been provided for plant breeding methods using “any other” maize plant comprising an EPSPS transgene linked to

a promoter and capable of being rendered male-sterile by treatment with glyphosate. The rejection was improperly maintained as set forth below.

(1) The Feng Declaration establishes the enablement of the claims

In response to the First Office Action, Appellants submitted the Declaration of Dr. Paul C.C. Feng, demonstrating the enablement of the claims. [Exhibit C] The studies described in the Declaration show that glyphosate-inducible male sterility is reproducible and not limited to the specific embodiments alleged by the Examiner.

Dr. Feng's Declaration describes *de novo* creation and analysis of transgenic plants having a glyphosate-inducible male sterile phenotype in multiple independent EPSPS transformation events. In one study described, five independent transformation events were obtained exhibiting inducible male sterility, three of which showed complete inducible sterility. [Exhibit C, para. 7]. The events comprised an expression-optimized CaMV 35S promoter upstream of an EPSPS transgene from *Agrobacterium tumefaciens* linked to a non-translated leader sequence from *Petunia hybrida* (hsp70). The five events were backcrossed (2x) into 4 different genotypes (87DIA4, LH59, LH195, and LH198). Acceptable glyphosate-inducible male sterility was observed for all five events transferred into the 87DIA4, LH195 and LH198 background. In an LH59 background, 4/5 events showed acceptable male sterility from V10/0.56 lb/a. The corresponding treatments for the control were all fertile. Greenhouse evaluations of plants at the R₀ stage also showed good male sterility in other backgrounds, including FBLL, LH172, LH244, and LH295. [Exhibit C, para. 7]

Dr. Feng's Declaration also describes numerous other plants prepared that exhibited inducible male sterility. In particular, plants were prepared using the constructs in Table 1, resulting in inducible male sterility. The results obtained demonstrate that glyphosate-inducible

male sterility can be used with multiple genotypes. While not every EPSPS event exhibits inducible male sterility, all that is relevant is that these plants can be prepared without undue experimentation, which has been demonstrated.

The studies described by Dr. Feng also confirm the reproducible mechanism that causes glyphosate-inducible male sterility. For example, Dr. Feng's Declaration shows that glyphosate arrests the maturation of microspore pollen cells, resulting in inviable pollen and male sterility. The impact of glyphosate was focused during the development of pollen; specifically during the development of the microspore mother cell, tetrad, and microspores. [Exhibit C, para. 5-6]. These studies also demonstrated that pollen with little or no expression of a glyphosate-resistant EPSPS transgene is susceptible to glyphosate, whereas pollen expressing high levels of resistant EPSPS is not. [Exhibit C, para. 6] In particular, immunolocalization studies showed that male fertile glyphosate-resistant plants displayed high expression of glyphosate-resistant EPSPS expression in the tapetum, microspore mother cell, tetrad and microspores, whereas plants exhibiting vegetative glyphosate tolerance and male reproductive intolerance (glyphosate-inducible male sterility), display low to no expression in the same tissues. [Exhibit C, para. 6]. As set forth in paragraph 5 of the Declaration, the results demonstrate that the description of the invention in the specification is correct and enabled. This, combined with the fact that tissue specific expression of transgenes was well known in the art (see Exhibits B and C), is more than adequate to demonstrate the enablement of the full scope of the claims.

(2) The evidence submitted has been improperly ignored

Dr. Feng concluded based on the studies carried out and descriptions in the specification that one of skill in the art in possession of the specification could “*readily prepare* transgenic plants with a *glyphosate-inducible male sterile phenotype using many different combinations*

of promoters and glyphosate resistant EPSPS transgenes without undue experimentation.” (emphasis added, **Exhibit C**, para. 9). The Examiner has provided no basis to doubt the truth of this statement or the working examples in Appellants specification. Instead, the Examiner has disregarded the evidence and applied his own *opinion*. This is improper.

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act (“APA”). 5 U.S.C. § 706(A), (E), 1994; *see also In re Zurko*, 59 USPQ 2d 1693 (Fed. Cir. 2001). In particular, the Federal Circuit has held that findings by the Board of Patent Appeals and Interferences must be supported by “substantial evidence” within the record pursuant to the APA. *See In re Gartside*, 203 F.3d 1305, 1314-15 (Fed. Cir. 2000). Thus, an Examiner’s position on Appeal must be supported by *substantial evidence* within the record in order to be upheld by the Board of Patent Appeals and Interferences. No *evidence* has been provided to doubt the enablement of Appellants’ claims. Rather the evidence submitted and working examples in the specification have been disregarded without support for doing so. This cannot stand in view of the requirements of the APA.

(3) The GG25 and GJ11 transformation events are not maize varieties and may be transferred into any maize genotype

With regard to the reference by the Examiner to “varieties” GG25 and GJ11, Appellants again respectfully note that GG25 and GJ11 are transformation events not maize varieties. As is described in the specification, these events may readily be transferred by plant breeding techniques including, for example, backcrossing, into other maize plant backgrounds. Accordingly, enablement is not limited to any given plant line. The GJ11 and GG25 events alone are therefore more than adequate to satisfy the enablement requirement.

In this regard, Appellants note that Example 14, at pages 92-93 of the specification, describes the introgression of the GG25 and GJ11 transformation events into elite inbreds and hybrids of maize. As set forth above, the GG25 and GJ11 events were each introgressed into elite inbred lines. Using the inbred lines, hybrid varieties were produced that contain these events and were demonstrated to possess the inducible male-sterility trait. The working examples therefore demonstrate full enablement of the claims.

With regard to the Examiner's suggestion on page 4 of the Final Office Action that GG25 and GJ11 are "admitted by Applicant to be elite events" it is noted that this is incorrect. Appellants are not aware of the meaning of an "elite event." The term "elite" has only been used in the context of elite inbred and hybrid maize varieties, which are understood in the art to constitute those varieties that have beneficial agronomic qualities, such as high grain yield. The GG25 and GJ11 events were introgressed in the working examples into elite maize varieties for agronomic purposes, but this has no relevance to the events themselves or the expression of the glyphosate-inducible male sterile phenotype generally.

(4) The embodiments acknowledged by the Examiner alone demonstrate enablement of the claims

The Examiner acknowledges that the claims are enabled for plant breeding using maize parent plants comprising an EPSPS transgene operably linked to a CaMV35S promoter, maize histone promoter or *Arabidopsis* histone promoter. These embodiments alone are sufficient to satisfy the enablement of the claims. The examples demonstrate that promoters capable of being used for production of glyphosate-inducible male sterile plants are not limited to any specific promoter. The three promoter examples acknowledged are from Cauliflower mosaic virus, maize and the plant *Arabidopsis*. These organisms are highly diverged. It goes without saying

that Cauliflower mosaic virus is highly diverged from the plants maize and *Arabidopsis*. Additionally, maize is a monocotyledonous plant, whereas *Arabidopsis* is a dicotyledonous plant. Again, these plants are therefore also widely genetically diverged from each other. The ability to use promoters from such diverse sources indicates that the glyphosate-inducible male sterility is not dependent on any specific promoter.

It is finally noted that the legal standard for enablement does not require that Appellants demonstrate enablement for all possible claimed iterations. Enablement must bear only a reasonable relationship to the scope of the claims. *In re Fisher*, 166 U.S.P.Q. 18, 24 (CCPA 1970). This is echoed in the MPEP: “[a]s long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied.” MPEP 2164.01(b) (citing *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (CCPA 1970)). In view of this standard and the substantial evidence presented herein above, Appellants respectfully submit that the full scope of the claims has been enabled. Removal of the rejection under 35 U.S.C. §112, first paragraph is thus respectfully requested.

X. CONCLUSION

It is respectfully submitted, in light of the above, that the pending claims are enabling under 35 U.S.C. § 112 first paragraph. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,



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Date: October 24, 2003

APPENDIX 1: APPEALED CLAIMS

83. (Amended) A method of plant breeding comprising the steps of:
- (i) planting in pollinating proximity seeds capable of growing into first and second parent maize plants, said first parent maize plant comprising a first EPSPS transgene, wherein said first parent plant is capable of being rendered male-sterile by treatment of said plant with glyphosate, and wherein said first plant is vegetatively and female reproductively tolerant to said treatment with the glyphosate;
 - (ii) cultivating said seeds to produce said first and second parent maize plants;
 - (iii) causing said first parent maize plant to be male-sterile by treating said first parent plant with said glyphosate;
 - (iv) allowing the second maize parent plant to pollinate the first parent maize plant; and
 - (v) harvesting seeds produced on the first parent maize plant.
84. (Amended) The method of claim 83, wherein said second parent maize plant is further defined as comprising a second transformation event, said second plant having vegetative tolerance to said glyphosate.
85. (Amended) The method of claim 84, wherein said second parent plant is still further defined as male reproductively tolerant to said glyphosate.
86. (Amended) The method of claim 85, wherein both said first and said second parent maize plants are treated with said glyphosate.
87. (Amended) The method of claim 86, wherein treating said first and second parent plants comprises an over-the-top application of said glyphosate.
96. (Amended) The method of claim 87, wherein treating comprises an over-the-top application of from 8 ounces per acre to 96 ounces per acre of glyphosate.
97. (Amended) The method of claim 83, wherein said treating is carried out between the V4 and VT stages of development.
98. (Amended) The method of claim 83, wherein the step of causing said first parent plant to be male sterile comprises an application of from 8 ounces per acre to 96 ounces per acre of glyphosate.

Promoter Elements Required for Developmental Expression of the Maize *Adh1* Gene in Transgenic Rice

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To define the regions of the maize alcohol dehydrogenase 1 (*Adh1*) promoter that confer tissue-specific expression, a series of 5' promoter deletions and substitution mutations were linked to the *Escherichia coli* β -glucuronidase A (*uidA*) reporter gene and introduced into rice plants. A region between –140 and –99 not only conferred anaerobically inducible expression in the roots of transgenic plants but was also required for expression in the root cap, embryo, and in endosperm under aerobic conditions. GC-rich (GC-1, GC-2, and GC-3) or GT-rich (GT-1 and GT-2) sequence motifs in this region were necessary for expression in these tissues, as they were in anaerobic expression. Expression in the root cap under aerobic conditions required all the GC- and GT-rich motifs. The GT-1, GC-1, GC-2, and GC-3 motifs, and to a lesser extent the GT-2 motif, were also required for anaerobic responsiveness in rice roots. All elements except the GC-3 motif were needed for endosperm-specific expression. The GC-2 motif and perhaps the GT-1 motif appeared to be the only elements required for high-level expression in the embryos of rice seeds. Promoter regions important for shoot-, embryo-, and pollen-specific expression were proximal to –99, and nucleotides required for shoot-specific expression occurred between positions –72 and –43. Pollen-specific expression required a sequence element outside the promoter region, between +54 and +106 of the untranslated leader, as well as a silencer element in the promoter between –72 and –43.

INTRODUCTION

Little is known of the mechanisms regulating tissue-specific expression in plants. In maize plants, the alcohol dehydrogenase 1 (*Adh1*) gene is expressed in the roots in response to anaerobic stress (Sachs et al., 1980), but it also has tissue-specific aerobic expression in scutellum, embryo, endosperm, root cap, and pollen cells (Freeling and Bennett, 1985). The gene is expressed under haploid genotype control in the pollen. To understand the mechanisms regulating tissue-specific expression of *Adh1*, a necessary step is to define the promoter regions controlling expression and the *trans*-acting factors that interact with them. This has not been possible using transgenic maize plants because of the absence of a routine transformation system. However, rice is transformed readily (Shimamoto et al., 1989) and is a suitable system for a detailed analysis of maize *Adh1* gene expression in different tissues (Kyoizuka et al., 1993; Terada et al., 1993).

In rice, the ADH enzyme occurs in the same tissues as in maize. One difference is that it is also found in mature green

rice leaves where it is induced weakly by anoxia (Xie and Wu, 1989). In transgenic rice, the maize *Adh1* promoter drives *Escherichia coli* β -glucuronidase A (*gusA*) (*uidA*) reporter gene expression in the roots, embryo, scutellum, endosperm, and pollen, similar to the pattern found in maize (Kyoizuka et al., 1991). Thus, all of the *trans*-acting factors required for developmental expression of *Adh1* appear to be present in rice and able to activate the maize promoter. More recently, Kyoizuka and Shimamoto (1992) observed expression from the maize promoter in the leaves of transgenic rice, suggesting that *trans*-acting factors in the nuclei of rice leaf cells also interact effectively with the maize *Adh1* promoter. Studies by Kyoizuka et al. (1991) indicated that all the *cis*-acting elements required for appropriate expression of the maize *Adh1* gene in rice are present in the region from –1096 to +106.

Anaerobic induction of the *Adh1* gene is regulated mainly at the level of transcription (Gerlach et al., 1982; Rowland and Strommer, 1986). Transient assays in maize suspension cell protoplasts revealed an anaerobic responsive element (ARE) between positions –140 and –99 (Walker et al., 1987; Olive et al., 1990, 1991). Two GC- and two GT-rich motifs within the ARE sequence were shown to be required for anaerobic induction in maize protoplasts (Olive et al., 1991), but the function of the ARE has not been tested in transformed plants. Specific binding of maize nuclear protein(s) to nucleotide

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sequences within the ARE has been demonstrated (Feri, 1990; Olive et al., 1991).

In this study, we identified *cis*-acting elements required for tissue-specific expression patterns of the maize *Adh1* gene in transgenic rice by examining the expression of a series of deleted and mutated promoters fused to the *gusA* reporter gene. Elements within the region -140 to +106 control expression in the root cap, endosperm, embryo, scutellum, shoots, and pollen of transgenic rice plants. We also demonstrated that the ARE (-140 to -99) conferred anaerobic inducibility in the roots of transgenic rice plants. Nucleotide sequences required for endosperm-, embryo-, and root cap-specific expression under aerobic conditions were coincident with the ARE. In contrast, expression in the scutellum, shoots, and pollen required elements proximal to the ARE. We mapped a region essential for shoot-specific expression between the ARE and the TATA box, and a region required for expression in pollen is located in the untranslated leader sequence.

RESULTS

The Promoter Region between -140 and -99 Functions as an Anaerobic Responsive Element in Transgenic Rice

The ARE (-140 to -99) identified in transfected maize protoplasts (Walker et al., 1987; Olive et al., 1990, 1991) has not been assayed in a transgenic plant system. Recently, Kyoizuka et al. (1991) found that the 1.1-kb maize *Adh1* promoter (construct AIGN in Figure 1) does confer anaerobically responsive expression on the *gusA* gene in roots of transgenic rice plants. We have now shown that a promoter truncated to -140 also confers anaerobic induction in the roots of R_1 seedlings. Whereas the level of induction observed varied (five- to 26-fold) in the transgenic lines tested, there was no significant difference between the full-length and truncated promoters. In all transformants, GUS expression was always observed in

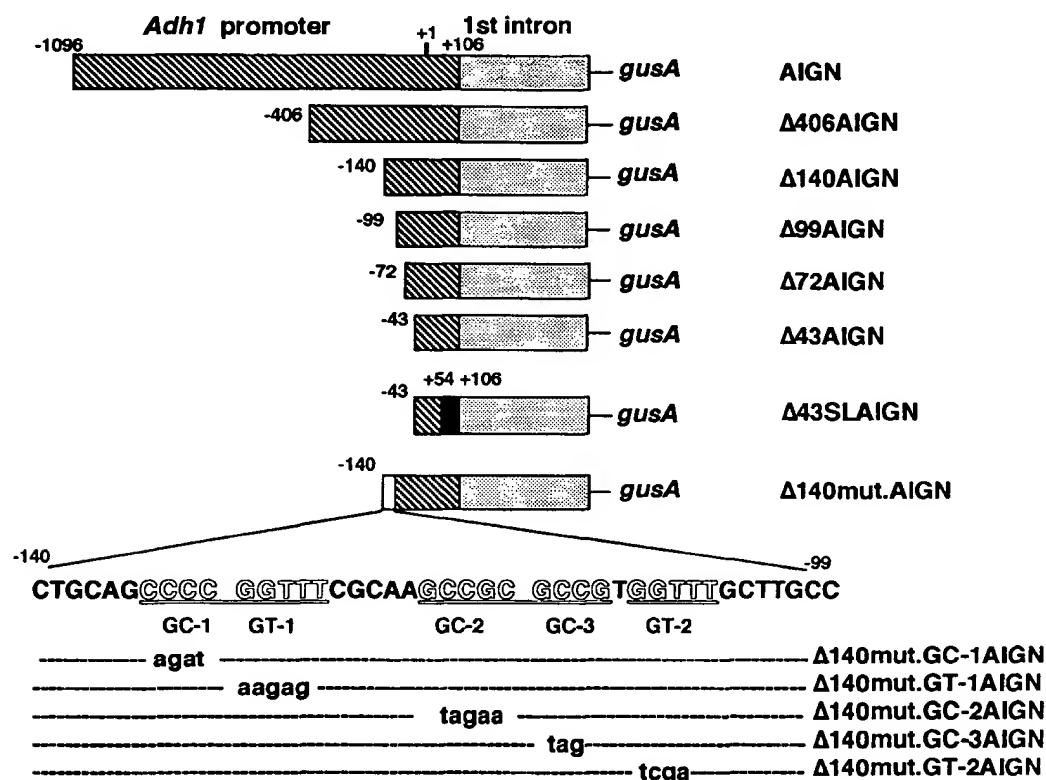


Figure 1. Maize *Adh1-gusA* Fusion Constructs Used for Rice Transformation.

All gene fusions contain the promoter plus leader region distal to +106 (hatched box) and the first intron (stippled box) from the maize *Adh1* gene placed upstream of the *gusA* gene and nopaline synthase transcription termination sequence. The 5' end point of each gene construct is given relative to the transcription start site of the maize *Adh1* gene. In $\Delta 43$ SLAIGN, the untranslated leader sequence from +53 to +106 was replaced by a fragment of pUC19 DNA of similar length (filled box). Constructs designated $\Delta 140$ mut.GC-1AIGN, $\Delta 140$ mut.GT-1AIGN, $\Delta 140$ mut.GC-2AIGN, $\Delta 140$ mut.GC-3AIGN, and $\Delta 140$ mut.GT-2AIGN each contain substitutions of 3 to 5 bp of DNA within the region -134 to -107. The wild-type sequence present in $\Delta 140$ AIGN is shown with the respective mutant sequences indicated below by lowercase letters.

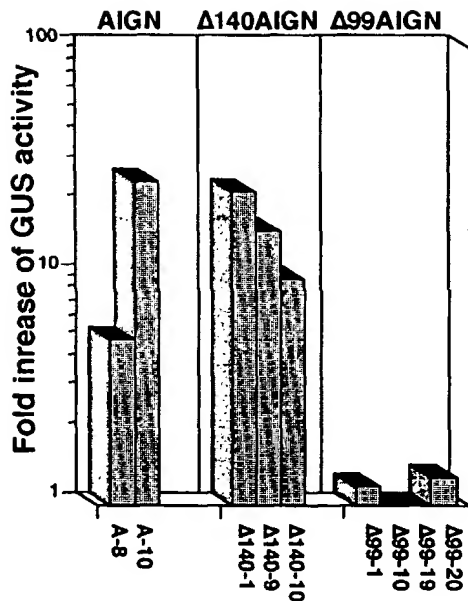


Figure 2. Anaerobic Induction of GUS Activity in the Roots of Transgenic Rice.

the elongation zone of the roots, paralleling the pattern of the endogenous rice *Adh1* gene activity under anoxia. In four different lines carrying the Δ99AIGN gene construct, no anaerobic induction of the *gusA* gene was observed in the roots (Figure 2). We concluded that the ARE sequence previously identified in homologous transient assays in maize protoplasts functions the same way in rice plants, conferring high levels of gene expression following anoxia.

Previous results obtained by Walker et al. (1987) and Olive et al. (1991) showed that the GC-1, GT-1, and GC-2 motifs and nucleotides -111 to -101, including the GT-2 motif (Figure 1), were critical for ARE function in maize protoplasts. In transgenic rice plants, substitution mutations in the region between -134 and -112, replacing the GC-1, GT-1, GC-2, and GC-3 sequence motifs, eliminated anaerobically responsive gene expression (Figure 3). The mutation of the GT-2 motif (-110

Plants transformed with the gene constructs AIGN (A-8 and A-10), Δ140AIGN (Δ140-1, Δ140-9, and Δ140-10), and Δ99AIGN (Δ99-1, Δ99-10, Δ99-19, and Δ99-20), respectively, were assayed for GUS-specific enzyme activity before and after anaerobic treatment. Roots from four to six seedlings were used for each assay. The fold-increase of GUS activity was calculated as the ratio of the mean specific enzyme activity following anoxia relative to that under aerobic conditions.

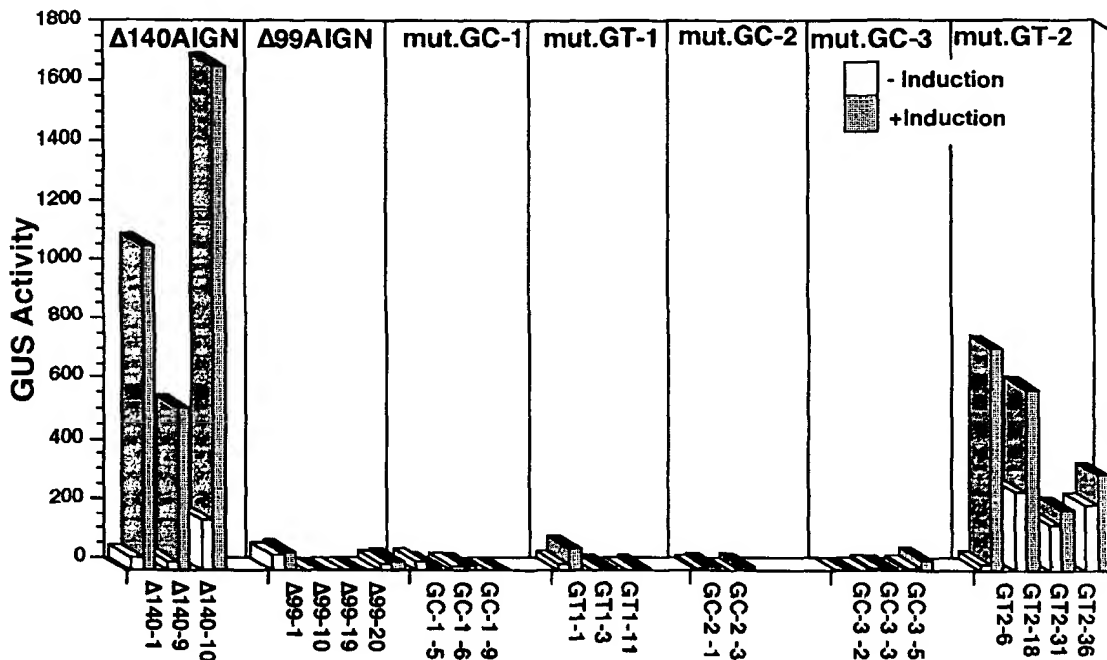


Figure 3. Effects of Substitution Mutations within the ARE Sequence on the Anaerobic Responsiveness of the *Adh1* Promoter in Rice Roots.

Extracts from the roots of rice plants transformed with the gene constructs Δ140AIGN, Δ140mut.GC-1AIGN (mut.GC-1), Δ140mut.GT-1AIGN (mut.GT-1), Δ140mut.GC-2AIGN (mut.GC-2), Δ140mut.GC-3AIGN (mut.GC-3), Δ140mut.GT-2AIGN (mut.GT-2), and Δ99AIGN were assayed for GUS-specific enzyme activity in aerobic conditions (open bars), or following anaerobic stress (filled bars). Assays were performed as given in the legend to Figure 2. Two to four independent transgenic lines (x-axis) were assayed in each case. GUS enzyme activity is shown on the abscissa in picomoles of 4MU produced per minute per milligram of protein. Gene constructs are shown at the top of the figure.

to -106) reduced the level of induction (Figure 3), whereas the mutation between -110 and -101 did abolish expression in the transient assay (Olive et al., 1991). This suggests that nucleotides between -106 to -101 are important for anaerobically responsive expression.

Nucleotides Downstream of -140 Contain All Elements Required for Developmental Expression in Rice

The *gusA* reporter gene driven by the maize *Adh1* promoter is expressed in transgenic rice plants in the shoot, root cap,

pollen, embryo, scutellum, and endosperm (Kyoizuka et al., 1991). To identify the *cis* elements responsible for expression in these tissues, we performed 5' deletions of the promoter region. The gene constructs used are shown in Figure 1.

The complete *Adh1* promoter (AIGN; Figures 4A, 4D, and 4G) and the *Adh1* promoter truncated to -140 ($\Delta 140$ AIGN; Figures 4B, 4E, and 4H) had identical patterns of expression—the gene was active in the same tissues, namely the root cap, pollen, embryo, scutellum, and endosperm. All the *cis*-acting elements necessary for expression in these tissues must be located downstream of -140 .

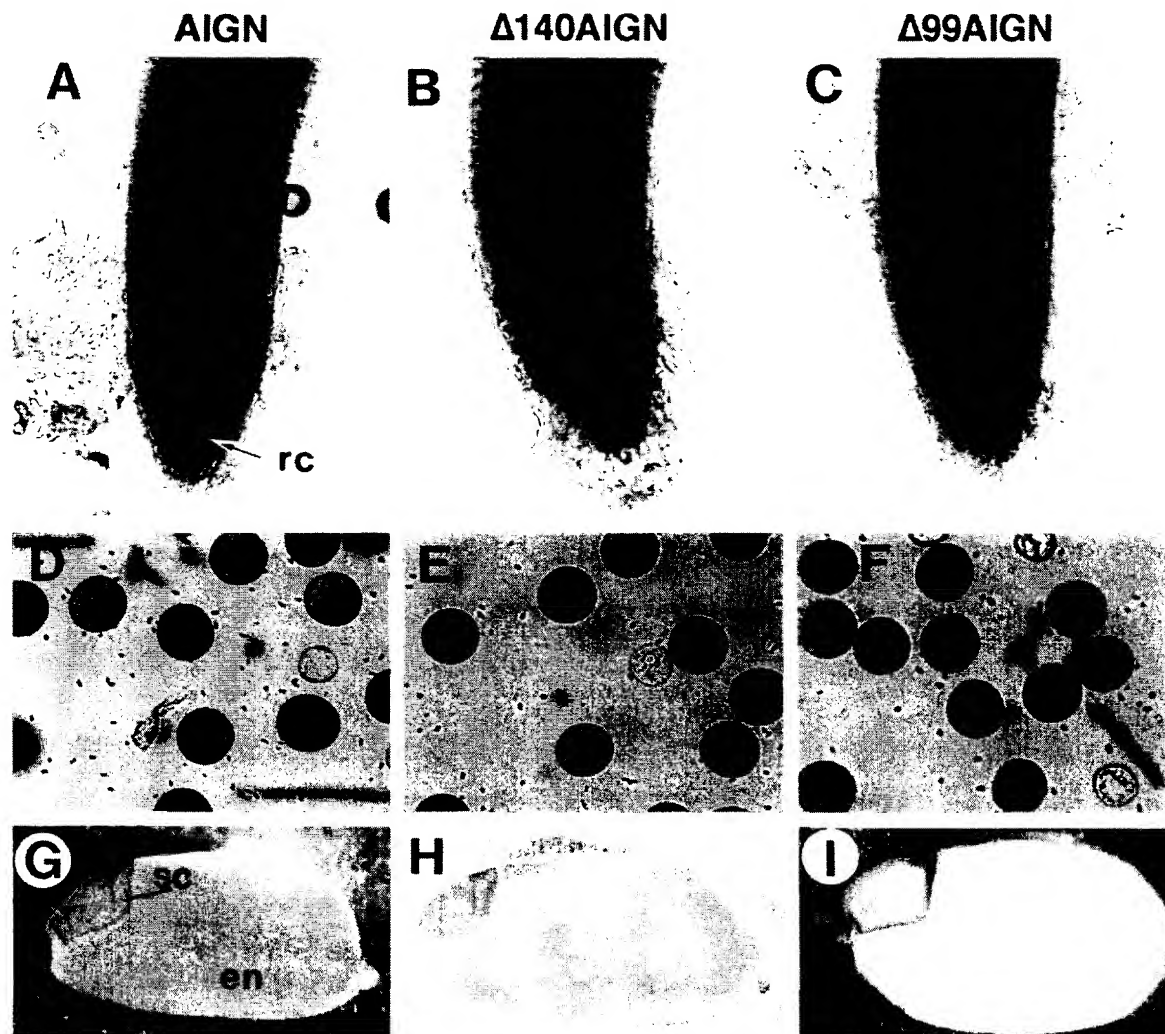


Figure 4. *gusA* Gene Expression of AIGN, $\Delta 140$ AIGN, and $\Delta 99$ AIGN in the Root Cap, Pollen, and Seeds of Transgenic Rice Plants.

(A) to (C) Expression of AIGN (A), $\Delta 140$ AIGN (B), and $\Delta 99$ AIGN (C) in the root cap.

(D) to (F) Expression of AIGN (D), $\Delta 140$ AIGN (E), and $\Delta 99$ AIGN (F) in pollen.

(G) to (I) Expression of AIGN (G), $\Delta 140$ AIGN (H), and $\Delta 99$ AIGN (I) in the seed.

em, embryo; en, endosperm; sc, scutellum; rc, root cap.

Expression of the Maize *Adh1* Gene in the Root Cap, Embryo, and Endosperm Requires Nucleotides Coincident with the ARE

Deletion of the nucleotides between -140 and -99 in the promoter abolished detectable expression in the root cap ($\Delta 99$ AIIGN, Figure 4C) and the endosperm (Figure 4I). Compared to the $\Delta 140$ AIIGN gene, the $\Delta 99$ AIIGN gene was expressed at a low level in the embryo (Figure 4I). Thus, the region between -140 and -99, as well as controlling the anaerobic response, was required for expression in the embryo. In contrast, GUS activity was observed in pollen (Figure 4F) and the scutellum (Figure 4I) in 10 independent $\Delta 99$ AIIGN transgenic lines, indicating that pollen-specific and scutellum-specific elements are downstream of -99. They are not contained within the ARE sequence.

To quantify the results of the histochemical analyses, we measured GUS activity fluorometrically in the embryo (including the scutellum) and endosperm of five independent $\Delta 99$ AIIGN plants and four independent $\Delta 140$ AIIGN plants (Figure 5). Both truncated promoters conferred *gusA* gene expression in the embryo, but the expression level of the $\Delta 99$ AIIGN construct in the embryo was only 5 to 20% (508 pmol 4-methylumbelliferone [4MU] produced per min per mg of protein) of the expression obtained with the $\Delta 140$ AIIGN construct (Figures 5A and 5B). This suggested that embryo-specific expression of the maize *Adh1* gene requires nucleotides downstream of -99 in addition to those between -140 and -99, which comprise the ARE region.

In the endosperm, the $\Delta 140$ AIIGN construct had a mean GUS activity of 775 pmol of 4MU per min per mg of protein (Figure 5C). GUS activity was not detectable in the endosperm of seeds from transgenic lines carrying the $\Delta 99$ AIIGN construct (Figure 5D), supporting the conclusion that nucleotides downstream of -140 are critical for endosperm-specific expression.

To determine which sequence motifs within the ARE are required for expression in the root cap, embryo, and endosperm under aerobic conditions, we examined the activity of five mutated promoters. Each mutant promoter contained a 3- to 5-bp substitution within the GC- and GT-rich sequence motifs of the ARE (Figure 1). In the absence of anoxic stress, the *gusA* gene was expressed highly in the root cap, embryo, and endosperm of plants transformed with $\Delta 140$ AIIGN (Figures 6A and 6B), which was the control construct in these experiments.

None of the five mutant promoters expressed GUS in the root cap (Figures 6D, 6F, 6H, 6J, and 6L). A low level of GUS activity was observed in the vascular and cortical tissues of roots carrying $\Delta 140$ mut.GT-2AIIGN, but no expression was observed in the root cap. This suggested that the GT-2 motif is also important for root cap expression. Deletion to -99 also abolished root cap expression (construct $\Delta 99$ AIIGN; Figure 6N). The mutation of each of the GC-1, GT-1, GC-2, and GT-2 sequence motifs reduced endosperm-specific gene expression to background levels, comparable to that obtained for $\Delta 99$ AIIGN (Figures 6C, 6E, 6G, 6K, and 6M). In contrast, GUS activity

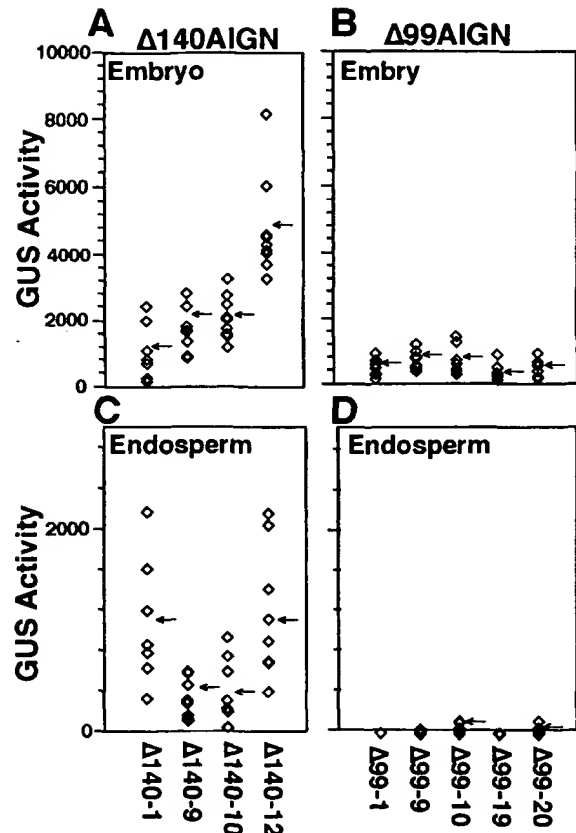


Figure 5. GUS Enzyme Activity in the Embryo and Endosperm of Plants Transformed with $\Delta 140$ AIIGN and $\Delta 99$ AIIGN.

Each point represents a single enzyme measurement obtained using seven to 10 seeds from each transgenic line and is indicated on the x-axis. GUS activity, shown as picomoles of 4MU produced per minute per milligram of protein, is represented on the y-axis. The arrows indicate mean GUS activity obtained for each independent transgenic line.

(A) and (C) Expression in the embryo (A) and endosperm (C) of four independent lines transformed with $\Delta 140$ AIIGN.

(B) and (D) Expression in the embryo (B) and endosperm (D) of five independent lines transformed with $\Delta 99$ AIIGN.

was detected in the endosperm of seeds carrying mutations in the GC-3 motif (Figure 6I). Expression in the embryo depended upon the presence of an intact GC-2 motif (Figure 6G) and perhaps also the GT-1 motif (Figure 6E). Significant expression was observed in the embryo even when the GC-1, GC-3, or GT-2 motifs were mutated (Figures 6C, 6I, and 6K, respectively), showing that these sequences are dispensable for embryo-specific expression.

In summary, these experiments showed that expression of the *Adh1* gene in the root cap, embryo, and endosperm requires the GT-1 and GC-2 sequence motifs and that expression

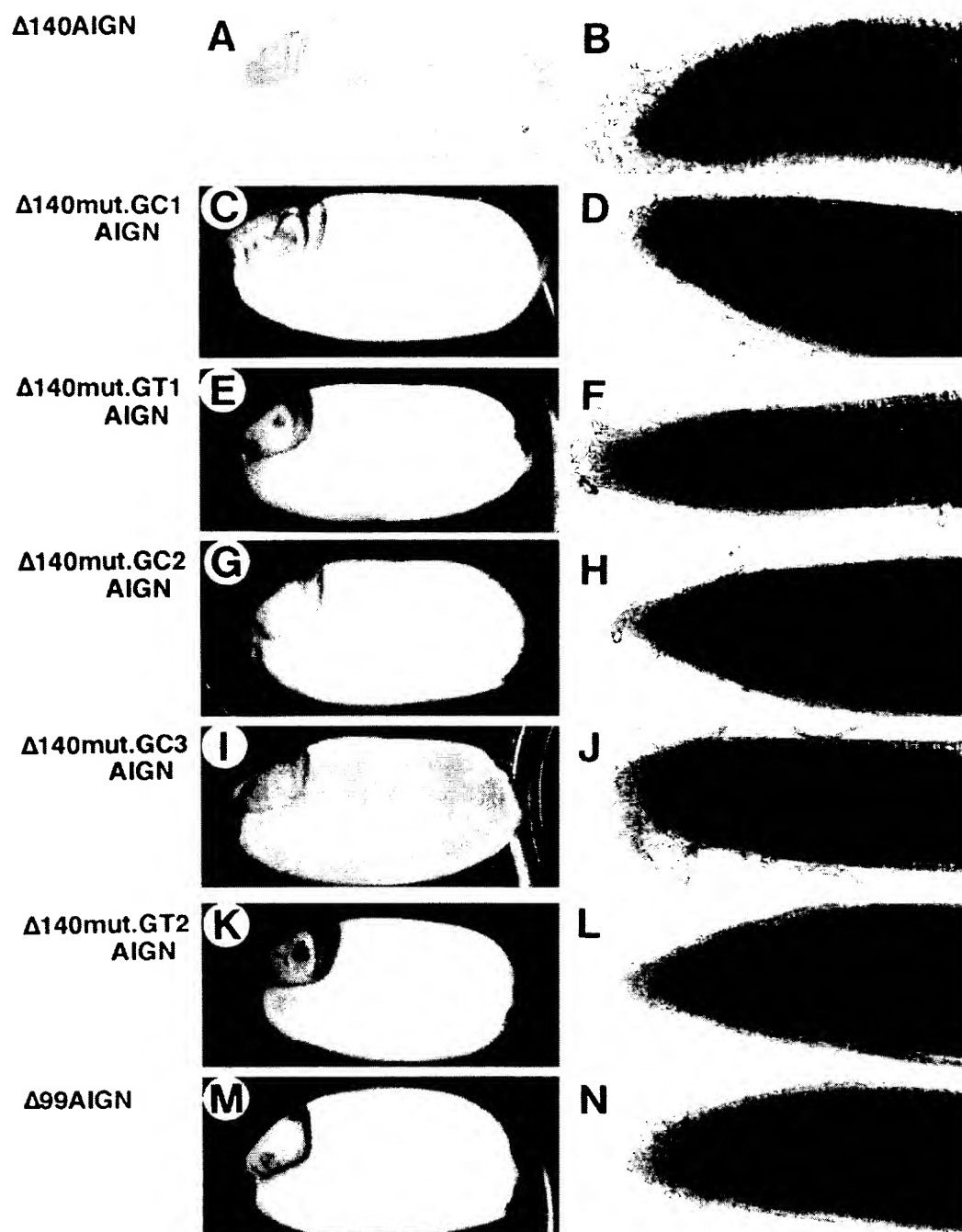


Figure 6. Expression of $\Delta 140\text{mut.AIGN}$ Gene Constructs in the Seeds and Root Caps of Transgenic Rice.

(A) and (B) $\Delta 140\text{AIGN}$ in the seed (A) and the root cap (B).
 (C) and (D) $\Delta 140\text{mut.GC-1AIGN}$ in the seed (C) and the root cap (D).
 (E) and (F) $\Delta 140\text{mut.GT-1AIGN}$ in the seed (E) and the root cap (F).
 (G) and (H) $\Delta 140\text{mut.GC-2AIGN}$ in the seed (G) and the root cap (H).
 (I) and (J) $\Delta 140\text{mut.GC-3AIGN}$ in the seed (I) and the root cap (J).
 (K) and (L) $\Delta 140\text{mut.GT-2AIGN}$ in the seed (K) and the root cap (L).
 (M) and (N) $\Delta 99\text{AIGN}$ in the seed (M) and the root cap (N).

in the endosperm also requires the GC-1 and GT-2 elements. Root cap expression requires all the motifs, including GC-3.

Nucleotides -72 to -43 of the Maize *Adh1* Gene Direct Shoot-Specific Expression in Transgenic Rice Plants

In maize, neither *Adh1* mRNA nor protein is detected in shoots and leaves. However, in rice, both the endogenous *Adh1* mRNA and ADH1 enzyme are present in aerobically grown shoots (Freeling and Bennett, 1985; Xie and Wu, 1989; Christie et al., 1991); this gene is induced weakly by anaerobic stress (Xie and Wu, 1989). Because the maize *Adh1* promoter is active in the shoots of rice plants (Kyoizuka and Shimamoto, 1992), we were able to use truncated promoters to identify the critical promoter region.

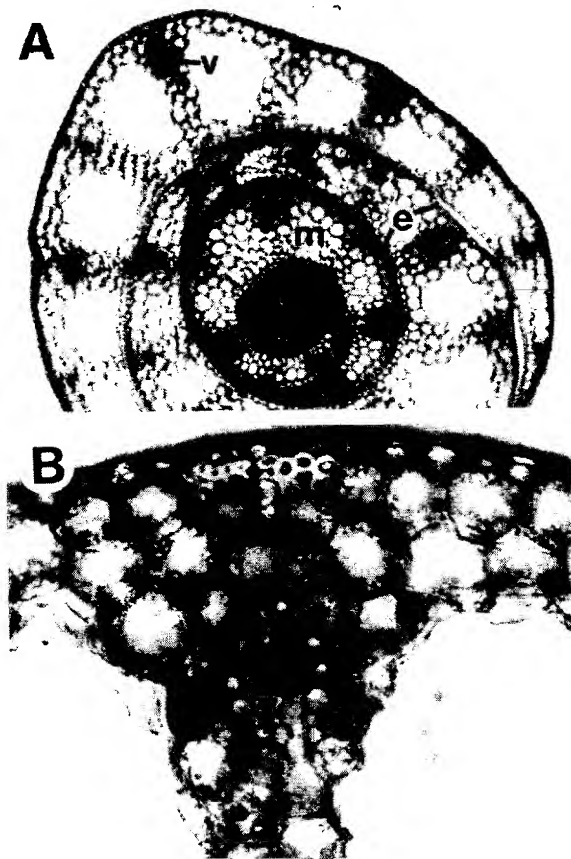


Figure 7. Expression of $\Delta 140AIGN$ in Shoots of Transgenic Rice.

(A) Histochemical staining for GUS activity in a transverse section of rice shoot obtained from a plant transformed with the $\Delta 140AIGN$ gene construct.

(B) Eightfold magnification of the vascular region shown in (A). e, epidermis; m, mesophyll; v, vasculature.

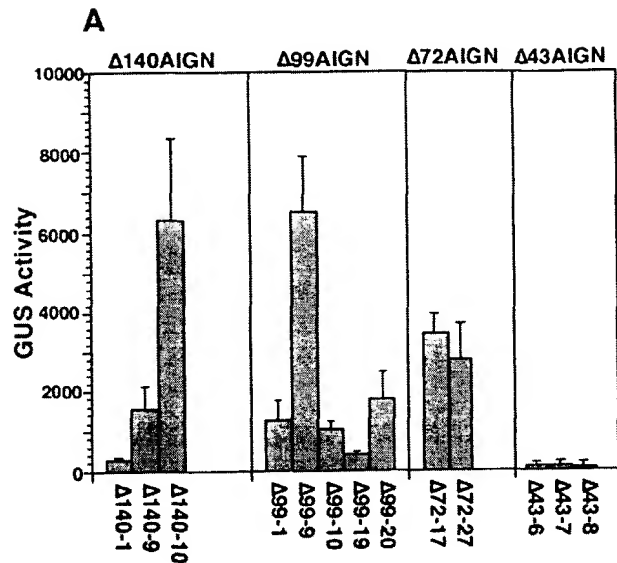


Figure 8. Activity of Several Truncated *Adh1* Promoters in Transgenic Rice Shoots.

Enzyme activity was measured in extracts from shoot sections ~2 mm long obtained from the base of five- to six-day-old seedlings. The x-axis indicates the transgenic line used. The values indicated show average GUS activity and standard deviation. Activities are in picomoles of 4MU produced per minute per milligram of protein.

In the shoot of transgenic rice, GUS activity was localized in the epidermis (Figure 7A) and the vascular cells (Figure 7B). Both constructs $\Delta 140AIGN$ and $\Delta 99AIGN$ showed a similar level of GUS activity in rice seedling shoots of ~0.3 to 6.5 nmol of 4MU per min per mg of protein (Figure 8). We concluded that all the *cis* elements required for shoot expression are downstream of -99.

Shoot expression was reduced by ~95% to ~150 pmol of 4MU per min per mg of protein when nucleotides between -72 and -43 were deleted (Figure 8). These data indicated a strong shoot-specific element in the region between or overlapping -72 to -43. Lu and Ferl (1992) showed that oligonucleotides containing this sequence repress maize *Adh1* promoter activity in cotransfected maize protoplasts, supporting a role for this region in positive regulation of the maize gene.

A Region of the Untranslated Leader of *Adh1* Is Required for Expression in the Pollen of Transgenic Rice Plants

Both $\Delta 99AIGN$ and $\Delta 140AIGN$ were expressed in pollen after the tetrad stage, and the expression level increased during pollen development (Figures 4E and 4F). Average GUS activity in anthers was not significantly different in plants expressing the $\Delta 140AIGN$ construct (3145 pmol of 4MU per min per mg of protein; Figure 9A) or the $\Delta 99AIGN$ construct (2182 pmol

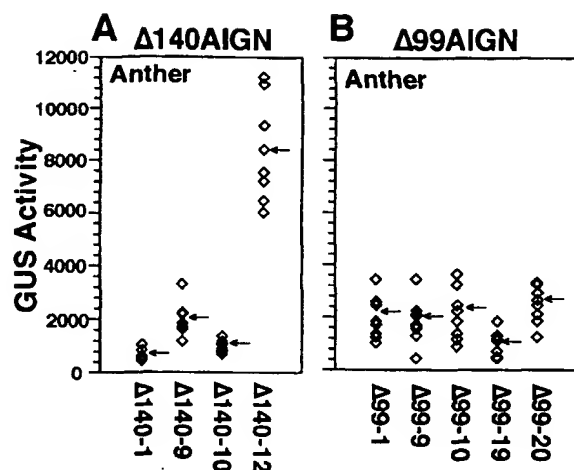


Figure 9. Quantitative Analysis of GUS Enzyme Activity in the Anthers.

GUS specific enzyme activities were determined for four independent lines transformed with $\Delta 140$ AIGN and five independent lines transformed with $\Delta 99$ AIGN. Each point represents a single enzyme measurement obtained using seven to 10 flowers from each transgenic line, indicated on the x-axis. GUS activity, shown as picomoles of 4MU produced per minute per milligram of protein, is represented on the abscissa. The arrows represent mean GUS activity obtained for each independent transgenic line.

(A) Anthers of plants transformed with $\Delta 140$ AIGN.

(B) Anthers of plants transformed with $\Delta 99$ AIGN.

of 4MU per min per mg of protein; Figure 9B), supporting the conclusion that a pollen-specific element is downstream of -99 .

We then examined the activity of the gene constructs $\Delta 72$ AIGN, $\Delta 43$ AIGN, and $\Delta 43$ SLAIGN (Figure 1). Both $\Delta 72$ AIGN and $\Delta 99$ AIGN gene constructs were expressed at comparable levels, ~ 2.5 nmol of 4MU per min per mg of protein (Figure 10). Expression increased significantly ($P = 0.001$) when the *Adh1* promoter was truncated to position -43 (10 nmol of 4MU per min per mg of protein; construct $\Delta 43$ AIGN, Figure 10), suggesting a pollen-specific silencer in the region between -72 and -43 . This element may be involved in repression of *Adh1* gene expression prior to meiosis when there is no detectable ADH enzyme activity (Stinson and Mascarenhas, 1985). It is unclear whether the pollen-specific silencer overlaps or is identical to the shoot-specific element also identified in this region of the *Adh1* promoter (Figure 8).

In contrast, substitution of 52 nucleotides within the leader region, from $+54$ to $+106$ with pUC118 DNA, reduced pollen-specific expression significantly ($P = 0.001$) by one to two orders of magnitude below that of $\Delta 43$ AIGN to less than 200 pmol of 4MU per min per mg of protein (Figure 10; compare $\Delta 43$ AIGN and $\Delta 43$ SLAIGN). These data were confirmed by staining of pollen for GUS enzyme activity. We concluded that a part of the leader sequence between $+54$ and $+106$ is responsible for high levels of expression of this gene in pollen.

This finding is consistent with data obtained by Kloeckener-Gruissem et al. (1992), who showed that maize lines containing a *Mutator* (*Mu3*) transposon-induced mutation upstream of the TATA box in the *Adh1* promoter have normal levels of ADH in the pollen but do not synthesize ADH in the sporophytic organs, roots, and scutellum. The reduction in ADH activity in the roots may be a result of increased distance of the ARE from the TATA box following *Mu3* insertion. In a later study, Dawe et al. (1993) showed that ADH1 activity in maize pollen is altered by the excision of a *Dissociation* (*Ds1*) transposable element from $+53$, suggesting that nucleotides around $+53$ in the leader sequence are important for pollen-specific expression.

Scutellum Expression Requires a Region Downstream of -43

When the *Adh1* promoter was truncated to -99 , GUS activity was observed in the scutellum of a number of transgenic rice

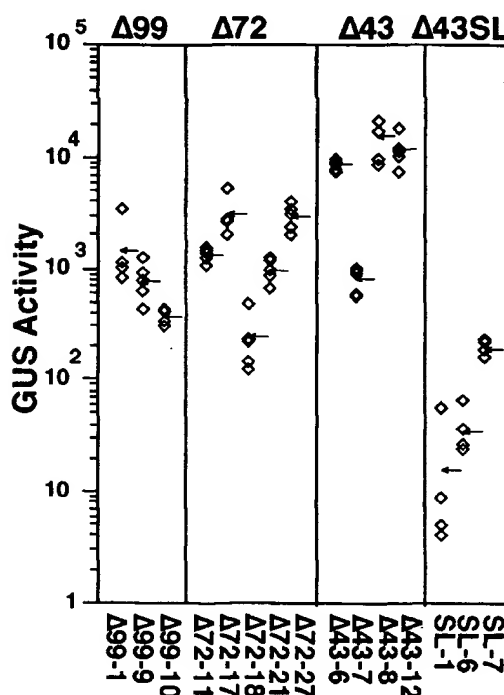


Figure 10. Identification of Regions Downstream of -99 in the *Adh1* Promoter Important for Expression in the Pollen.

The gene constructs $\Delta 99$ AIGN ($\Delta 99$), $\Delta 72$ AIGN ($\Delta 72$), $\Delta 43$ AIGN ($\Delta 43$), and $\Delta 43$ SLAIGN ($\Delta 3$ SL) were assayed for their activity in pollen of transgenic rice plants. Quantitative determination of GUS enzyme activity, shown on the abscissa as picomoles of 4MU produced per minute per milligram of protein, was determined for three to five independent transgenic lines carrying each construct (x-axis). Each point represents the activity in isolated pollen grains from a single flower. Five flowers from each transgenic line were used in the analysis. Arrows indicate the mean GUS activity for each transgenic line.

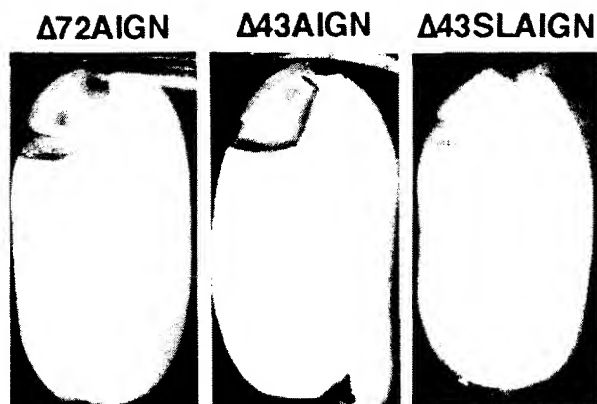


Figure 11. Identification of Regions Downstream of -99 in the *Adh1* Promoter Important for Expression in the Scutellum.

The gene constructs $\Delta 72AIGN$, $\Delta 43AIGN$, and $\Delta 43SLAIGN$ were assayed for their activity in the seeds of transgenic rice.

plants (Figure 4I), indicating that some elements important for scutellum-specific expression in rice are downstream of -99. The seed sections showed different levels of GUS activity in the region of the scutellum, and it is possible that promoter elements upstream of -99 changed the pattern of expression

when present (Figures 4G and 4H compared with Figure 4I), but we are unable to make firm conclusions on the basis of the present data. The three chimeric gene constructs $\Delta 72AIGN$, $\Delta 43AIGN$, and $\Delta 43SLAIGN$ (Figure 1) were also expressed in the scutellum of transgenic plants (Figure 11). Because none of the promoter mutations abolished expression in this tissue, we cannot map precisely the regions essential for scutellum-specific expression of the maize *Adh1* gene. Because the *Adh1* sequence contained in these constructs was relatively short and the only unmutated region was the 100-bp sequence between -43 and +54 (or in the intron 1 sequence included in these gene constructs; Figure 1), this region may contain the elements for scutellum-specific expression.

DISCUSSION

We have identified a number of *cis* elements in the maize *Adh1* promoter between -140 and +106 that control expression under aerobic conditions and in response to anoxia. These results are summarized in Figure 12. Various combinations of these sequence elements interact with their binding proteins to regulate expression both in various tissues in the plant and in response to the metabolic demands of anaerobic stress, this response being primarily in the cells of the root.

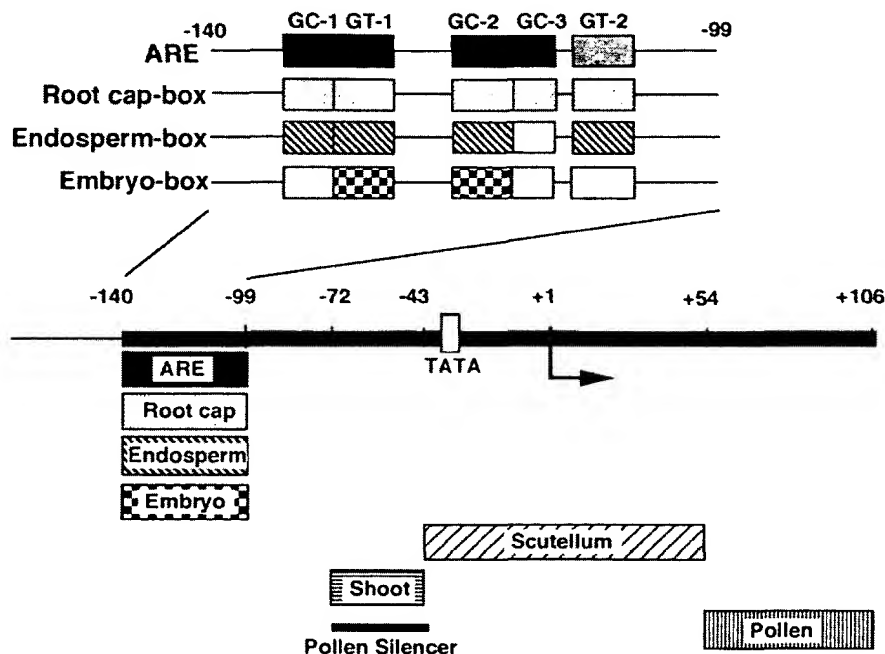


Figure 12. Summary of the *cis*-Acting Elements in the Maize *Adh1* Promoter.

The *Adh1* promoter is shown (black line) with the TATA box indicated (open box) and numbering relative to the start of transcription. Above, the positions of the GC-1, GT-1, GC-2, GC-3, and GT-2 motifs within the region between -140 and -99 of the *Adh1* promoter are indicated. Sequence motifs that are important for anaerobic responsiveness (ARE), root-cap expression, endosperm-, and embryo-specific expression are indicated by filled, stippled, hatched, and gridded boxes, respectively. Below, the extent of regions important for activity of the maize *Adh1* promoter in rice embryos, shoots, scutella, and pollen are also indicated.

The ARE sequence, previously determined in maize transient assays to lie between -140 and -99 , also functions in rice plants to give anaerobic expression. Sequences within the ARE are also important for expression in the root cap, endosperm, and embryo under aerobic conditions. The GC-3 element within the ARE is required for expression only in the root. Because expression in the "aerobic" root cap and the anoxic root requires all of the same *cis*-acting elements within the ARE, we suggest that expression in root cap may actually be an anaerobic response rather than a true example of developmental expression. Measurements of oxygen concentration within roots using an oxygen electrode show that the lowest concentration can occur in the root cap (Armstrong et al., 1993), and so it is likely that under normal aerobic conditions, the root cap is hypoxic. As the oxygen concentration drops, other root cells become hypoxic and the *Adh1* gene is activated.

Expression in other tissues requires different combinations of the various sequence motifs within the ARE. The GT-1 and GC-2 motifs are critical for expression in endosperm and embryo, as well as being required for the anaerobic response in the root. In some tissues, one or more of the motifs seems to be dispensable. For example, GC-1, GC-3, and GT-2 are not needed for embryo expression, and GC-3 is not needed for endosperm expression. Possibly, different proteins bind to the ARE motifs in different tissues. If the proteins binding to the ARE motifs in embryo and endosperm cells are the same as in anaerobic roots, in the future, we may be able to understand why the GT-1 and GC-2 motifs are sufficient for embryo cell expression but not for expression in other tissues. One possible explanation is that additional tissue-specific post-translational modifications of binding proteins, such as phosphorylation, or protein-protein interactions are involved. Alternately, there may be additional promoter elements downstream of -99 required for expression in the embryo and endosperm that bind tissue-specific factors. Gain-of-function experiments may show whether the motifs contained within the ARE are sufficient for tissue-specific expression of *Adh1*.

Sequence elements between the ARE and the TATA box are important for shoot-specific expression and for pollen silencing. Nucleotides -79 to -44 of the maize *Adh1* promoter comprise a tract of extreme homopurine/homopyrimidine asymmetry that is S1 hypersensitive (Fert et al., 1987). The nucleotide sequence CCCTCC(A/T)CCCY(G/C)TC(G/C)TTTC from -79 to -61 of the maize *Adh1* promoter (Dennis et al., 1984) is conserved in the same region of the rice *Adh1* gene (A. Liu, personal communication) and may represent a shoot-specific element. In contrast, the rice *Adh2* gene, which is expressed primarily in roots, does not contain this consensus sequence, although it does have a tract of homopurine/homopyrimidine asymmetry in this region. Specific mutation experiments should show if precisely the same element is required for shoot expression and pollen silencing.

Elements downstream of the TATA box and located in the transcribed leader are necessary for pollen expression. A factor binding to these elements may still interact with TATA box

binding protein, but our data show that it does not require any interaction with promoter elements upstream of -43 . A pollen box (PB) core motif (TGTGGTT) is present in the promoters of the tomato genes *Lat52*, *Lat56*, and *Lat59*, and functional assays have shown that the GG doublet in the PB core is critical for expression in pollen (Twiss et al., 1991). The *Adh1* untranslated leader sequence contains a related sequence motif AGTGGAT between $+63$ and $+69$ that may be functionally equivalent to the PB motif. The rice *Adh1* gene, which is expressed in pollen (A. Liu, personal communication; Xie and Wu, 1989), also contains the sequence GGTGGTT in the leader region. In addition, the sequence T(T/G)YGGGATYYG (GAT box), immediately downstream of the putative PB core and ~ 94 to 113 bp downstream of the TATA boxes, is present in the leader sequences of the maize *Adh1* (Dennis et al., 1984), rice *Adh1* (A. Liu, personal communication), and pearl millet *Adh1* (Gaut and Clegg, 1991) genes. This 12-mer sequence is not present in the 5' untranslated leader of the maize *Adh2* (Dennis et al., 1985) or the rice *Adh2* (Xie and Wu, 1990) genes, which are not expressed in pollen. This sequence may prove to be critical for pollen-specific *Adh1* gene expression in monocot plants.

The identification of a distinct silencer and a positive regulatory element involved in pollen-specific expression of *Adh1* is significant in view of the timing of ADH1 enzyme accumulation during male gametogenesis and suggests that two distinct molecular switches may regulate this key biological clock. Prior to meiosis, repressor protein(s) may bind to the negative regulatory element between -72 and -43 to prevent *Adh1* gene expression. In haploid pollen, the level of this repressor may be reduced, derepressing the *Adh1* gene and permitting expression regulated by an element in the untranslated leader region. The conservation of pollen-specific elements, in particular the GAT box, in the untranslated leader region of *Adh1* genes expressed in pollen poses interesting questions on how this positive regulation occurs. The GAT box may be the binding site for a transcription factor.

On the other hand, this element may be necessary for post-transcriptional regulation. For example, the GAT box sequence may increase mRNA stability or effect the association of *Adh1* mRNA with polyribosomes. There is some evidence for post-transcriptional control of maize *Adh1* in the male gametophyte (Kloeckener-Gruissem et al., 1992). In the revertant *Adh1-3F1124r17*, excision of the *Mu3* transposable element caused an 18-bp deletion in the TATA box region. The revertant contains normal levels of *Adh1* mRNA in the pollen but reduced ADH1 enzyme activity in these cells when compared to its parent plant.

Our study reveals many similarities between maize and rice in promoter elements and suggests there is considerable conservation of the mechanisms regulating anaerobic induction and tissue-specific expression. Comparisons of the promoters and leaders of several monocot *Adh1* genes (e.g., pearl millet, rice, and wheat) suggest that these findings may also hold true for the genes of other monocots. Although more can be done to define the specific *cis*-acting sequences involved in expression in the shoot, scutellum, and pollen and in isolating specific

transcription factors, regulatory mutants will reveal details of the intricate signal transduction pathways.

METHODS

Plasmid Construction

All plasmids were constructed using standard recombinant DNA techniques and were verified by nucleotide sequence analysis. All clones described in this study are based on the plasmid pIGN, which contains the maize alcohol dehydrogenase 1 (*Adh1*) first intron sequence cloned upstream of the *Escherichia coli* β -glucuronidase A (*gusA*) (*uidA*) gene and the nopaline synthase transcription termination sequence.

The construction of plasmids pAIGN (Kyoizuka et al., 1991), pIGN, p Δ ARE AIGN, and pARE AIGN (Olive et al., 1990) have been reported elsewhere. For convenience and to be consistent with the naming of other clones in our promoter deletion series, we have called the plasmids p Δ ARE AIGN and pARE AIGN, referred to by Olive et al. (1990), p Δ 99AIGN and p Δ 140AIGN, respectively. The plasmids p Δ 72AIGN and p Δ 43AIGN were constructed by subcloning the *Adh1* promoters from -72 to +106 and from -43 to +106 by 5' deletion and the addition of *Sall* linkers, respectively, into the polylinker of pIGN. Plasmids p Δ 72AIGN and p Δ 43AIGN have *Adh1* promoter 5' termini at positions -72 and -43, respectively, and 3' termini at position +106. To construct p Δ 43SLAIGN, the *Adh1* promoter from position -43 to -53 was first subcloned as a *PstI*-*DdeI* fragment from p Δ 43AIGN into pUC118 digested with *PstI* and *HincII*. A 45-bp *AluI* spacer fragment of pUC18 DNA was then introduced into an end-filled *XbaI* site downstream of the *Adh1* fragment to produce p Δ 43SL. Finally, the truncated *Adh1* promoter plus pUC18 spacer DNA were subcloned as a *Sall*-*BamHI* fragment from p Δ 43SL into pIGN. Plasmids containing substitution mutations within the region -140 to -99 of the *Adh1* promoter were constructed by cloning annealed complementary oligonucleotides directly into the *PstI* site upstream of the truncated *Adh1* promoter in p Δ 99AIGN.

Production of Transgenic Rice Plants

Transgenic rice plants (cvs Nipponbare and Kinuhikari) were produced by electroporation of protoplasts isolated from embryogenic suspension cultures. Various *Adh1*-*gusA* fusion genes were cotransformed into protoplasts with a hygromycin-resistance (*hph*) gene. Electroporation and selection of hygromycin-resistant calli were performed according to Shimamoto et al. (1989). Transgenic calli expressing the *gusA* gene were selected among hygromycin-resistant calli by staining with X-gluc and transferred to the regeneration medium. Regeneration of plants from transformed calli was as described previously (Kyoizuka et al., 1987; Shimamoto et al., 1989).

Analysis of *gusA* Expression

GUS activity was assayed in roots and shoots of 5- to 6-day-old seedlings and in whole anthers, pollen, and embryos and endosperm of mature seeds. Both histochemical staining and fluorometric measurement of GUS specific enzyme activity were carried out according to Jefferson et al. (1987) with minor modifications. Twenty percent methanol

was included in the X-gluc solution and the reaction mixture to reduce the endogenous activity (Kosugi et al., 1990).

For histochemical staining, all the plant material was incubated in an X-gluc solution for 18 to 24 hr at 37°C. After staining, the shoot sections were incubated in 70% ethanol to remove chlorophyll. The terminal regions of roots were examined on whole roots because of the importance of maintaining the integrity of the root caps.

GUS enzyme activity was measured fluorometrically for extracts of root tips ~3 mm long that were excised from a single seedling. Shoot sections ~2 mm long obtained from the base of a seedling were used to determine GUS activity in shoots. To measure the enzyme activity in the embryo and endosperm separately, embryos were excised from the seeds with fine forceps. A disc of endosperm tissue ~1 mm long was prepared from the remaining seed.

Anaerobic Treatment

Anaerobic treatment was performed as described previously by Kyoizuka et al. (1991). Five- to 6-day-old seedlings were submerged in sterilized water in the dark for 24 hr at 30°C. N_2 was bubbled into water continuously for 15 min before the anaerobic treatment. After the treatment, GUS activity in roots of each seedling was measured. The fold-increase in GUS activity was calculated as the ratio of the mean specific enzyme activity following anoxia relative to that under aerobic conditions.

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Dissection of a pollen-specific promoter from maize by transient transformation assays

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Abstract

We have previously reported the isolation and characterization of a gene (Zm13) from *Zea mays* which shows a pollen-specific pattern of expression. Stably transformed tobacco plants containing a reporter gene linked to portions of the Zm13 5' flanking region show correct temporal and spatial expression of the gene. Here we present a more detailed analysis of the 5' regions responsible for expression in pollen by utilizing a transient expression system. Constructs containing the β -glucuronidase (GUS) gene under the control of various sized fragments of the Zm13 5' flanking region were introduced into *Tradescantia* and *Zea mays* pollen via high-velocity microprojectile bombardment, and monitored both visually and with a fluorescence assay. The results suggest that sequences necessary for expression in pollen are present in a region from –100 to –54, while other sequences which amplify that expression reside between –260 and –100. The replacement of the normal terminator with a portion of the Zm13 3' region containing the putative polyadenylation signal and site also increased GUS expression. While the –260 to –100 region contains sequences similar to other protein-binding domains reported for plants, the –100 to –54 region appears to contain no significant homology to other known promoter fragments which direct pollen-specific expression. The microprojectile bombardment of *Tradescantia* pollen appears to be a good test system for assaying maize and possibly other monocot promoter constructs for pollen expression.

Introduction

Microsporogenesis in flowering plants involves a complex program of gene expression [17, 18]. While a large number of genes are expressed in

the developing microspore and pollen grain, only a small fraction of these genes are pollen-specific, i.e. are expressed just in pollen and not in other tissues of the plant [21, 28, 29]. Pollen-expressed genes from several plants have been isolated and

analyses carried out of their promoters [1, 24, 25, 26, 27]. We have isolated and characterized a pollen-specific genomic clone from maize, designated Zm13 [7]. Zm13 has sequence homology to a pollen-expressed gene from tomato (LAT52) [23]. A set of 5' promoter deletions of Zm13 have been previously shown to correctly direct the temporal and spatial expression of a reporter gene in stably transformed tobacco plants [5]. A 375 bp Zm13 sequence from -314 to +61 relative to the transcription start site at +1 retains in transgenic tobacco plants the ability for correct tissue specificity and temporal specificity of expression as in maize [5]. We report here a more detailed dissection of the pollen-specific promoter of Zm13 by transient expression of a reporter gene, using high-velocity microprojectile bombardment to transform mature pollen. These results have been previously reported in abstract form [19].

Materials and methods

Plant material

Greenhouse-grown plants of *Tradescantia paludosa* L. were used as the source of pollen and leaf tissue for the assays. Tobacco NT1 suspension culture cells were grown and prepared for bombardment as described [30]. *Tradescantia* pollen was collected and stored at -20 °C before use. Maize (W-22) pollen (2-3 days before dehiscence) was collected from cut tassels and used fresh.

Plasmid constructions

All of the constructs containing Zm13 [7, 8] promoter fragments were created from the original plasmid pCIB391 [5] (gift of Lyle Crossland). This plasmid (now called -0.1-GUS-NOS) contains a 1062 bp promoter fragment of Zm13 extending from a *Hind* III site at -1001 to a *Ppu* MI site at +61 of the untranslated 5' region, ligated in front of the β -glucuronidase (GUS) [11] reporter gene and nopaline synthase 3' terminator.

The vector backbone for this plasmid was Bluescript SK- (Stratagene). PCR was utilized to create the -260, -100, -54, and -38 promoter constructs by the selection of upstream primers which generated fragments starting with those specific nucleotides of the promoter. These primers all contained sequences which created a *Hind* III site at the 5' end of the resulting promoter fragment. The downstream primer in each case hybridized within the GUS gene, generating the appropriate promoter fragment plus a *Xba* I-*Sma* I region of the pUC19 polylinker which contained a *Bam* HI site. The PCR reactions were performed under normal conditions (30 μ l reactions containing 10 ng template DNA, 1 μ M each primer, 200 μ M of each dNTP, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂, 0.01% gelatin, 1 U of AmpliTaq DNA polymerase (Cetus), layered over with 30 μ l of mineral oil). Twenty-five cycles of PCR were performed (92 °C for 2 min, 60 °C for 2 min, 72 °C for 3 min) on a Coy Tempcycler (Coy Laboratory Products, Ann Arbor, MI). The resulting products were extracted with an equal volume of chloroform, identified by electrophoretic separation on a 1% agarose-3% NuSieve (FMC Bioproducts) gel, the appropriate bands cut out, and the DNA purified using a GeneClean kit (Bio 101, La Jolla, CA). After digestion with *Hind* III and *Bam* HI the bands were ligated into a similarly digested pBS+ (Stratagene, La Jolla, CA) plasmid which contained GUS-NOS. The resulting clones were sequenced to ensure fidelity of the generated promoter region.

To create the plasmid with the Zm13 3' terminator, a 468 bp *Pvu* II fragment (from +611 to +1079; see [7]) which contains the polyadenylation signals and site, was subcloned into the *Sma* I site of pUC13 such that the 5' end of the insert was nearest the *Eco* RI end of the pUC13 polylinker. The insert was then removed as a *Sac* I/*Kpn* I fragment (the *Kpn* I site is in the insert at +1057, thus reducing the size of the 3' fragment from 468 to 446 bp) and cloned between the GUS gene and the 35S terminator of the 35S-GUS-35S plasmid. The GUS gene and Zm13 terminator were removed as a *Bam* HI/

Kpn I fragment and subcloned into pBS+. The promoter fragment was generated and ligated as above.

The plasmid pRT102GUS, containing the *Escherichia coli* β -glucuronidase gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and 35S terminator [20] (gift of Erwin Heberle-Bors) was used as a control.

Pollen transformation

Plasmids containing the Zm13 promoter fragments fused to the GUS reporter gene and with either the NOS or Zm13 3' terminators were used to coat tungsten particles (M10) which were then bombarded into pollen grains using an improved helium-driven biolistic device [30]. The DNA coating procedure with the 'flying disc' method was as described [30]. Bombardments were done at a gas pressure of 10.5 MPa and a flight distance of 1 cm. The distance between the rupture membrane and flying disc was 6 mm, and the distance from the disc to the shelf containing the Petri dish with pollen was 80 mm.

Sterile plastic Petri dishes (60 mm \times 15 mm) containing 3 ml of pollen medium (0.01% H_3BO_3 , 10 mM $CaCl_2$, 0.05 mM KH_2PO_4 , 0.1% yeast extract [Difco], and 10% sucrose) [16] with 1% agar were overlaid with 300 μ l of liquid pollen medium containing 30 μ g/ml chloramphenicol (to prevent bacterial contamination) and 4 mg of *Tradescantia* pollen. The pollen suspension was spread evenly over the surface of the medium using a miniature glass bacterial-type spreader and turntable. Bombardments were done within 5–15 min of spreading the pollen. The plates were kept at room temperature for 12–16 h prior to assay for GUS activity. Histochemical staining with 0.7 ml of the X-gluc reagent [10] was carried out in the Petri dishes at 37 °C overnight.

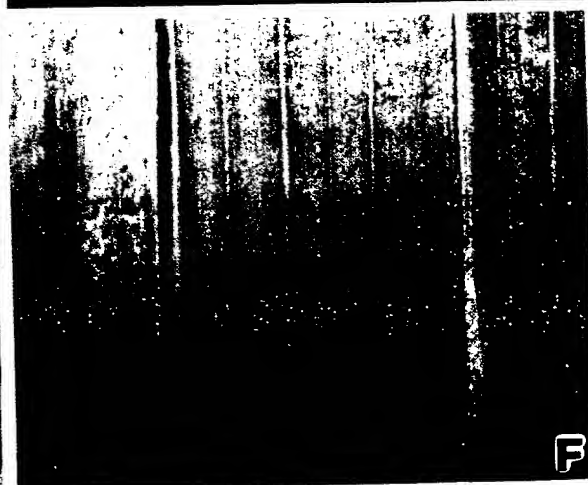
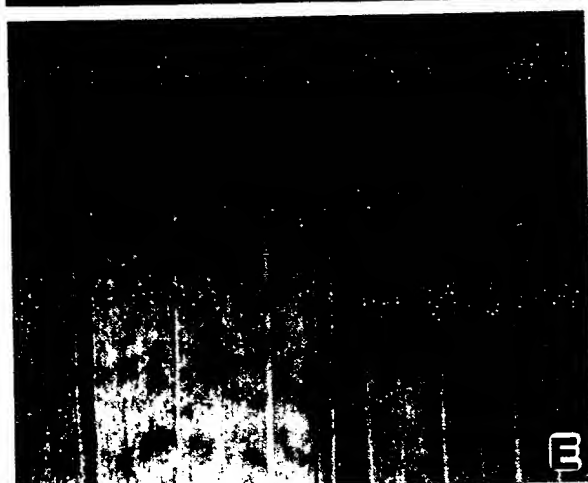
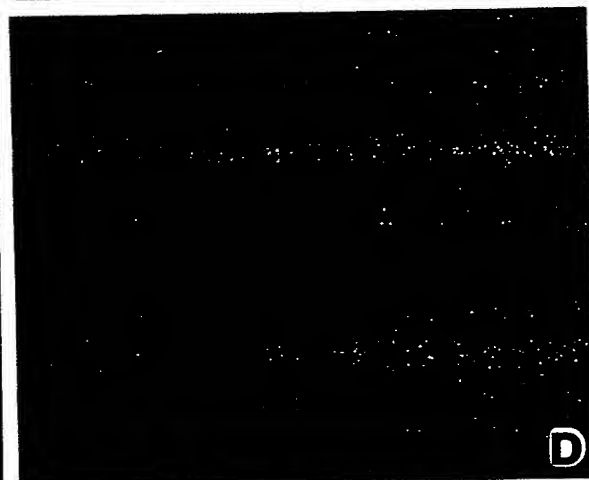
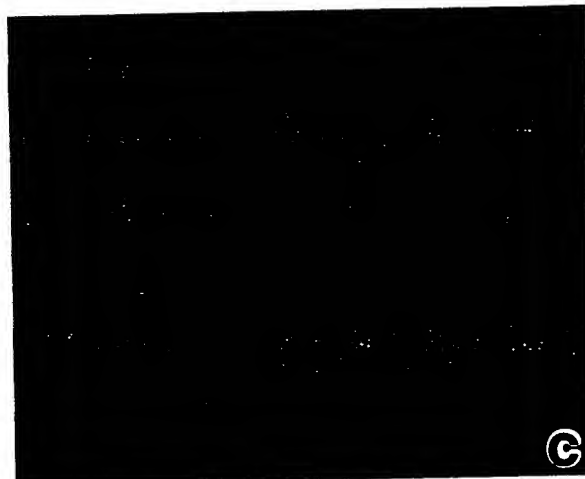
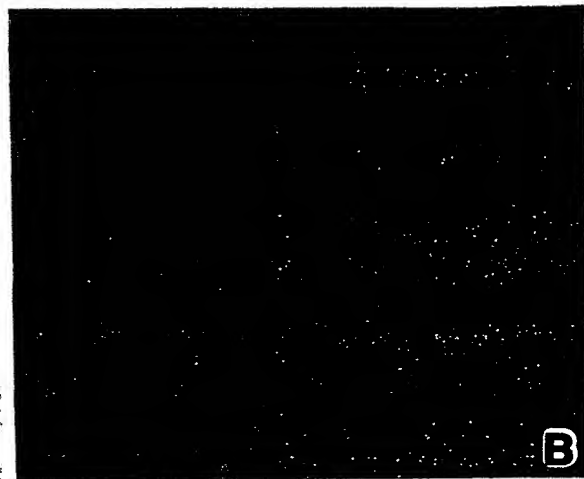
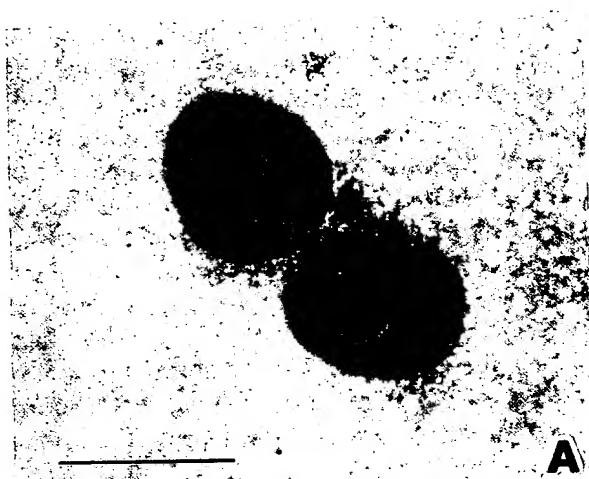
For bombardments done with leaf tissue, young *Tradescantia* leaves were cut and soaked in sterile water containing 50 μ g/ml of chloramphenicol for at least 15 min. The leaves were cut lengthwise along the mid vein and in sections of about 4–5 cm. The leaf pieces were placed 5 to a plate with

the lower surfaces facing up, on top of two 7.0 cm circles of sterile Whatman No. 1 filter paper in a Petri dish with 2.4 ml of sterile H_2O containing 50 μ g/ml chloramphenicol. After bombardment the plates were kept under lights in the laboratory for 36–48 h. The leaf sections were transferred to small tubes with 2 ml of the X-gluc reagent for histochemical staining. Tissue was stained overnight at 37 °C. The tissue was fixed in FAA (10% formalin, 5% glacial acetic acid, 42.5% ethanol) [9], and cleared in 70% ethanol and finally in 95% ethanol to remove green pigment. Blue cells or groups of cells were counted under a microscope.

Fluorometric assay for β -glucuronidase

GUS activity was measured by the fluorogenic assay developed by Jefferson [10] as briefly described here. Germinated pollen, along with the agar medium, was transferred to ice-cold tubes followed by addition of 0.2 ml of 4 \times cold GUS extraction buffer (200 mM sodium phosphate pH 7.0 containing 40 mM β -mercaptoethanol, 40 mM Na_2EDTA , 0.4% sodium lauryl sarcosine, and 0.4% Triton X-100). A pinch of polyvinyl pyrrolidone was added. The samples were ground in a mechanically operated teflon homogenizer until greater than 90% of pollen tubes were broken. The homogenate was centrifuged at 10000 \times g for 10 min at 4 °C. The supernatants were collected and assayed for GUS activity. Corrections were made for the volume of liquid remaining in the agar pellets. In control experiments with agar medium to which known amounts of enzyme activity were added, recoveries of about 87–90% were obtained.

An aliquot of the supernatant fractions obtained above, was incubated with 0.8 ml of 1 mM 4-methylumbelliferyl- β -D-glucuronidase (Sigma) in 1 \times GUS extraction buffer. The tubes were vortexed and incubated at 37 °C. At 0, 30, 60, 90, 120, and 180 min after incubation, 0.1 ml aliquots were removed and added to tubes containing 1.5 ml of 0.2 M Na_2CO_3 . The concentrations of the product formed, methyl umbelliferone, were



determined by using a fluorescence spectrophotometer (Perkin Elmer Model MPF-3L) with excitation at 365 nm and emission at 455 nm. The instrument was calibrated using 1 μ M methyl umbelliferone (Sigma). GUS activity is defined as the amount of methyl umbelliferone produced expressed as nmol/min per mg pollen.

Results

Pollen transformation

Tradescantia pollen was used for the bombardments because of its experimental convenience and advantages. Under long-day greenhouse conditions the plants flower continuously throughout the year, and *Tradescantia* pollen can be stored frozen for long periods of time while retaining its viability. The pollen germinates (typically 85–95%) within 5 min of inoculation on germination medium, and displays an even rate of pollen tube growth for several hours. In contrast, maize pollen does not retain its viability if frozen, bursts easily even on optimal medium, and there are large variations in germinability and tube growth of pollen collected on different days. These are obvious drawbacks considering the requirement that pollen be incubated for several hours after bombardment to determine GUS expression. Maize pollen can, however, be transiently transformed by microprojectile bombardment with an appropriate pollen promoter (Fig. 1A), but it is not an experimentally satisfactory system. *Tradescantia* pollen is an excellent system for these purposes given its highly reproducible germination and growth characteristics.

While this work was in progress Twell *et al.* [22] reported that chimeric genes under the control of a pollen-specific promoter from tomato

were transiently expressed in tobacco pollen after introduction by high-velocity microprojectile bombardment. The expression appeared to retain the pollen specificity observed in stably transformed transgenic tobacco plants. Our work extends these findings by showing similar results with a monocot pollen-specific promoter and pollen. Constructs containing the GUS gene under the control of the Zm13 promoter or subfragments that are active in pollen are unable to induce GUS expression in leaves or NT1 tobacco suspension cells as measured by cytochemical staining, while a construct using the CaMV 35S promoter can cause transient expression of the GUS gene in these tissues (see Figs. 1E, F for *Tradescantia* leaves; NT1 cells not shown). The microprojectile bombardment system using *Tradescantia* pollen thus reproduces the specificity of expression that is seen in the intact maize plant, and seems to be a good test system for assaying promoter constructs for pollen expression.

To obtain a relative measurement of the ability of the various Zm13 promoter fragments to direct the expression of the GUS gene, pollen bombardment by the different constructs was analyzed for GUS activity by the fluorometric assay. The various constructs used are shown in Fig. 2 and the results obtained are presented in Table 1. The most active promoter fragment was the –260 construct. A larger fragment, the –1001 construct, was only 50% as active as the –260 construct. Promoter activity was drastically reduced with the –100 construct being only 6% as active as the –260 construct. It is interesting that when the NOS terminator is replaced with the Zm13 terminator, activity of the –100 promoter construct is increased from 6% to about 30%. Promoter fragments less than 100 bp from the start of transcription were unable to induce GUS activity in pollen from chimeric genes. The CaMV

Fig. 1. Pollen and leaf transformed by microprojectile bombardment and processed as described in Materials and methods. Equal amounts of pollen were applied to each plate; apparent differences in pollen density reflect isolated uneven regions on a plate. A. Maize pollen, 2–3 days before dehiscence, bar = 100 μ m; B–D. Mature *Tradescantia* pollen, bar in C = 100 μ m; E, F. *Tradescantia* leaves (each leaf section is approximately 5 mm wide); transformed by bombardment with the following constructs: A, –1001-GUS-NOS; B, –260-GUS-NOS; C, –100-GUS-NOS; D, E, 35S-GUS-35S; F, –54-GUS-NOS (other Zm13 promoter constructs produced similar results).

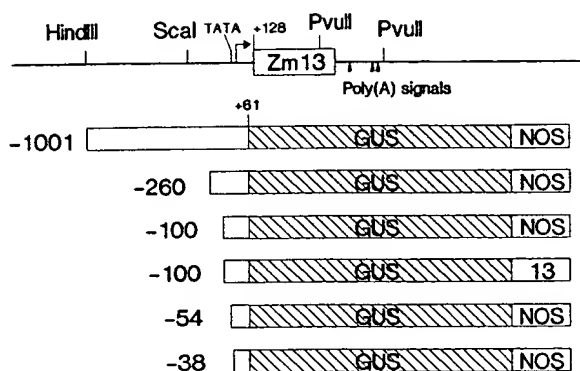


Fig. 2. Partial restriction map of Zm13 (top) showing position of promoter fragments and terminator (*Pvu* II fragment) used in chimeric constructs for microprojectile bombardment.

35S promoter has very weak activity in pollen, just about 2% that of the -260 construct.

The various promoter constructs were used in 6 different sets of bombardments over a period of about a year, and the intensity of X-gluc staining monitored visually through a microscope. The relative activities of the different constructs determined qualitatively by the intensity of blue staining were the same as those determined by the quantitative fluorometric analyses. The results described are thus very reproducible.

The activity of all the promoter constructs was assayed by the histochemical staining procedure after bombardment of leaves and NT1 tobacco suspension cells. The 35S CaMV promoter was very active in these vegetative tissues. About 2000–3000 blue groups of cells were seen per Petri plate of NT1 cells (data not shown) and several hundred blue spots were present in each leaf segment (Fig. 1E). None of the pollen-specific promoter constructs showed activity in these vegetative tissues, although on occasion, a few blue NT1 cells were seen and on some leaf sections a few trichomes, guard cells and epidermal cells stained blue. Whether this was caused by contamination of the gun by the 35S construct or a slight leakiness of the pollen promoter is not yet clear. Carryover due to contamination from previous bombardments is a problem but can be kept to a minimum by cleaning the gun chamber between bombardments.

Table 1. Fluorometric determinations and relative GUS expression produced by various constructs in transiently transformed *Tradescantia* pollen.

Construct	nmol MU/min/ mg pollen	Relative activity
- 1001-GUS-NOS	0.483 ± 0.085	51.9
- 260-GUS-NOS	0.929 ± 0.151	100.0
- 100-GUS-NOS	0.059 ± 0.006	6.4
- 100-GUS-13	0.375 ± 0.053	(29.7) ¹
- 54-GUS-NOS	0.009 ± 0.001	1.0
- 38-GUS-NOS	n.d.	0.0
35S-GUS-35S	0.023 ± 0.003	2.4

n.d. = not detected.

¹ Experiment performed only once with this construct (three replicates). The relative expression for the - 100-GUS-13 construct reflects comparison with other values from that experiment only. All other values are the averages of two experiments with 3 replicates per experiment.

Discussion

We previously showed that as little as 314 bp of 5' flanking DNA of a pollen-specific maize gene (Zm13) is able to effect correct temporal and spatial expression of a chimeric gene in transformed tobacco plants [5]. We have now further localized the promoter regions necessary for expression in pollen utilizing transient expression of transformed chimeric genes following microprojectile bombardment.

Particle gun transformation of *Zea mays* and germinating *Tradescantia* pollen with maize pollen-specific promoter constructs results in transient expression of the introduced reporter gene (Fig. 1A–D) as has been shown for tobacco by Twell *et al.* [22]. The improved helium microparticle gun used in these experiments is much superior to its gunpowder-charge predecessor in that the extreme damage to the center of the sample is avoided, and the target tissue is struck and transformed in a very uniform fashion. In our hands, bombardment with this newer device does not seem to have any inhibitory effects on *Tradescantia* pollen germination or tube growth compared to unbombarded controls. As a result, the reproducibility of experiments remained high in spite of being performed at widely spaced inter-

vals. As controls, we have bombarded *Tradescantia* leaf sections (Fig. 1E, F), as well as cultured tobacco cells. Because of the efficiency of the helium gun, the excellent properties of *Tradescantia* pollen, and the reproducibility of the results, a reference gene internal control was not considered necessary.

Measurements of the relative expression of the GUS gene directed by various Zm13 promoter fragments (Table 1) show that a -260 to +61 promoter fragment gives the maximum expression in pollen. Expression of GUS in constructs controlled by a larger (-1001 to +61) fragment is approximately half that of the -260 promoter fragment, suggesting the presence of a negative regulatory element in this region. Interestingly, we have located from sequence searches of the DNA database, a 174 bp region of the Zm13 5' flanking region (-686 to -513) which is 87% homologous to a portion of the 3' untranslated region of a maize 19 kDa zein gene [13]. The homologous sequence is in the reverse direction relative to the Zm13 gene, and contains at least four individual inverted repeats of 6-9 base pairs. Genomic Southern analysis using a portion of this region as a probe has shown that this sequence is highly repetitive in this genome [13]. Whether this region is involved in the decreased expression associated with the -1001 fragment, or whether its presence is anything other than a random consequence of rearrangement in the corn genome is unknown, but would be interesting to examine.

The -260 to +61 promoter fragment showed GUS expression 15-20-fold above that observed with a -100 to +61 fragment, indicating the presence of a quantitative element(s) essential for efficient expression in pollen in the -260 to -100 region of the 5' flanking region of Zm13. However, the -100 to +61 fragment still correctly directs expression in pollen but not in leaves or NT1 cells, albeit at a reduced level. Since further deletion of the promoter region to -54 results in almost total loss of GUS expression, this suggests that the pollen-specific element(s) is present in the -100 to -54 region.

These results are similar to those presented by

Twell *et al.* [25], who showed that a tomato promoter fragment containing a TGTGG motif could act as an enhancer to a truncated pollen promoter or to a heterologous CaMV 35S promoter. There are two such motifs, one in each orientation, within the -260 to -100 region of Zm13. This motif is similar to the common G-box protein-binding region which has been found associated with numerous plant promoters, e.g. maize and *Arabidopsis* Adh genes [2, 15], light-regulated genes [3, 4, 14], ABA-regulated genes [6], and is similar to the Hex sequence located in the promoter regions of the CaMV 35S and *Agrobacterium* nopaline synthase (NOS) genes [12]. It is not yet known if these enhancer-like regions of Zm13 may act in a pollen-specific fashion, or if their action is simply to amplify the expression exerted by an additional pollen-specific region. One possible model would be that the -260 to -100 region acts in an enhancer-like fashion to amplify the expression of a pollen-specific core promoter present in the -100 to -54 region. We are directing our efforts at answering these questions.

Comparison of the sequence of the -100 to -54 core promoter region with other minimal promoter regions of pollen- and anther-specific genes reveals no obvious consensus sequences, although some of the regions shown to contain the elements necessary for pollen specificity are fairly small [25, 27, this report]. Perhaps added unknown factors such as size, positional requirements, or additional *cis*-acting elements play a role in regulating the pollen-specific expression directed by these fragments.

To examine the possibility that sequences 3' to the coding region play a role in expression, the NOS terminator of the minimal pollen-specific promoter (-100 to +61) was replaced by a region of Zm13 containing the polyadenylation signals and site. GUS expression in the transient assay was increased roughly 6-fold over that directed by the fragment with the NOS terminator. This could be due to several factors, including enhanced stability or processing of the message in the transformed pollen. Clearly, the identity of *cis*-elements and other factors involved in pollen specificity of

expression is not yet determined and our current work is being directed towards this end.

Acknowledgements

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Michael Spencer, Rita Mumm,
J. Jefferson Gwynn, David McElroy and
Michael A. Stephens

Group Art Unit: 1638

Examiner: Kruse, David H.

Serial No.: 09/698,789

Atty. Dkt. No.: DEKM:157USC1

Filed: October 27, 2000

**For: METHOD FOR PLANT BREEDING (AS
AMENDED)**

**CERTIFICATE OF MAILING
37 C.F.R. § 1.8**

I hereby certify that this correspondence is being deposited
with the U.S. Postal Service as First Class Mail in an
envelope addressed to: Commissioner for Patents,
Washington, D.C. 20231, on the date below:

01/31/03
Date

Robert E. Hanson

DECLARATION OF DR. PAUL FENG UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

I, PAUL C.C. FENG, HEREBY DECLARE AS FOLLOWS:

1. I am currently employed by Monsanto Company, the parent company of DeKalb Genetics Corporation, with the title of Research Scientist. I have a Ph.D. in Biochemistry from North Dakota State University. I have been conducting research in the area of agricultural biotechnology for over 21 years. I am the author of 46

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manuscripts in peer-reviewed journals and a frequent speaker at national meeting in areas of weed science, plant physiology and agricultural biotechnology.

2. I am familiar with the subject matter disclosed and claimed in the above-referenced patent application.

3. I understand that the Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application has rejected the claims as not being supported by adequate information in the specification regarding the ability to produce transgenes that confer a glyphosate-inducible male sterile phenotype in maize.

4. Therefore, I am providing the present Declaration to submit further data that demonstrate the enablement of the invention claimed in the current patent application.

5. Studies carried out under my supervision have shown the broad applicability of the use of glyphosate-resistant EPSPS coding sequences to engineer plants exhibiting vegetative tolerance and male reproductive sensitivity to glyphosate (glyphosate-inducible male sterility). These studies were initiated with the goal of obtaining plants having sufficient expression of glyphosate-resistant EPSPS in vegetative tissues to provide vegetative tolerance to glyphosate, but having little or no expression in male reproductive parts, so that the plants exhibit a glyphosate-inducible male sterile phenotype. This strategy is set forth on pages 78 and 79 of the above-referenced patent application. There it is indicated, and our studies have confirmed, that the glyphosate-inducible phenotype is due to this expression profile.

6. Histological studies carried out on transgenic maize at Monsanto Company have shown that glyphosate arrests the maturation of microspore pollen cells, resulting in inviable pollen and male sterility. The studies indicate that the impact of glyphosate is focused at specific stages of pollen development; during the development of the microspore mother cell, tetrad, and microspores. Experiments indicate that, once microspores begin to mature (~V14 stage), glyphosate is no longer effective.

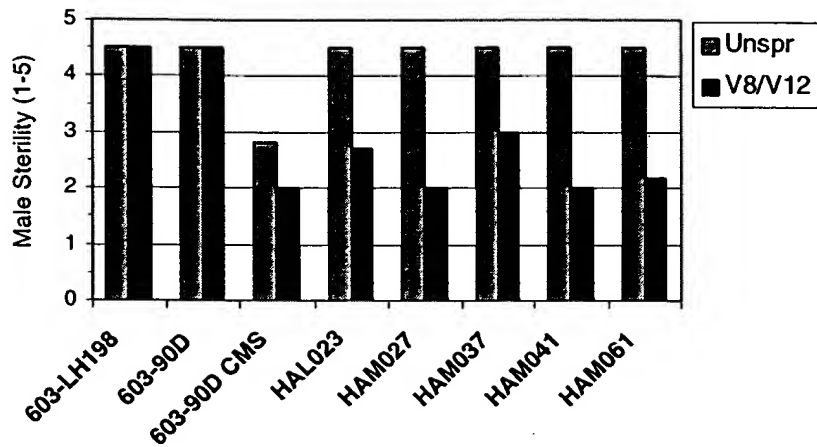
Studies carried out by myself and others at Monsanto Company have demonstrated that pollen with little or no expression of a glyphosate-resistant EPSPS transgene is susceptible to glyphosate, whereas pollen expressing high levels of resistant EPSPS is not. In particular, immunolocalization studies carried out at Monsanto Company demonstrated that male fertile glyphosate-resistant plants display high expression of glyphosate-resistant EPSPS expression in the tapetum, microspore mother cell, tetrad and microspores. In contrast, plants exhibiting vegetative glyphosate tolerance and male reproductive intolerance (glyphosate-inducible male sterility), display low to no expression in the same tissues. These results demonstrate that the inducible male-sterile phenotype is dependent on the level of expression of glyphosate-resistant EPSPS in these male tissues. This is further supported, for example, by the description of others of glyphosate-induced male sterility in cotton (U.S. Patent Application No. 20020111272) and the ability to render pollen inviable with the herbicide chlorsulfuron in *Brassica napus* plants (U.S. Patent No. 6,476,291) .

7. The *de novo* creation and analysis of transgenic plants having a glyphosate-inducible male sterile phenotype that was carried out under my supervisions can be summarized as follows:

Engineering of glyphosate-inducible male sterility:

Five homozygous transformation events (HALO23, HAM37, HAM027, HAM041, HAM061) were prepared using a transgene construct comprising an expression-optimized CaMV 35S (CaMVe35S) upstream of a non-translated leader sequence from *Petunia hybrida* (hsp70) linked to a glyphosate-tolerant EPSPS from *Agrobacterium tumefaciens* (CP4 EPSPS). Plants homozygous for each transformation event were examined for glyphosate-induced male sterility and yield in 6 locations. Controls contained the NK603 transformation event in corn line LH198 (603-LH198) and 90DJD28 (603-90D) backgrounds as well as a NK603-CMS (cytoplasmic male sterile) line in the 90DJD28 (603-90D CMS) background. The NK603 transformation event comprises a CP4 EPSPS coding sequence and confers vegetative and male reproductive tolerance to glyphosate. LH198 and 90DJD28 are proprietary inbred maize lines of Monsanto Company.

Five treatments were carried out on the plants: unsprayed, hand detasseled by removal of 3 top leaves, V8 spray at 0.75 lb/a glyphosate, V12 spray at 0.56 lb/a, and a double spray at V8fbV12. All plants were treated with a V4 spray of 0.48 lb/a to insure that they were resistant to glyphosate. A comparison of the male sterility rating of the unsprayed control and the double sprays (V8fbV12) is presented in the figure below. Full male sterility with no anther extrusion is achieved at a rating scale of 2, while full fertility is achieved at a rating of 5.



The results showed that both NK603 controls (603-LH198 and 603-90DJD28) were fully male fertile (4.5 to 5.0 score) with the double spray. The NK603-CMS line in 90DJD28 showed incomplete male sterility (2.8 score) as expected having the CMS trait, for which sterility was improved by the double glyphosate spray treatment. Three out of the 5 events prepared with the e35S/hsp70/CP4 transgene (HAM027, HAM041, HAM061) showed glyphosate-induced full male sterility (2.0 score), while the remaining two events showed incomplete male sterility. Single sprays (V8 or V12) showed pollen shed at 90% silking, suggesting that a higher rate may be needed to generate full male sterility from a single spray in late stage development. These results demonstrated that all of the CaMVe35S/hsp70/CP4 EPSPS events examined in this study exhibited a glyphosate effect on male sterility, three of five events exhibited complete sterility. The results confirm the ability to reproducibly generate glyphosate-inducible male sterile plants, as evidenced by the three events exhibiting full glyphosate-induced male sterility. These results are representative of a larger body of events that demonstrated glyphosate-induced male sterility.

Inbred genotype performance. The 5 events examined above were backcrossed (2x) into 4 different genotypes (87DIA4, LH59, LH195, and LH198). The ability to induce male sterility was evaluated by applications of a single spray from V6-V12 at 0.56-0.75 lb/a glyphosate. The corresponding NK603 inbred in the same genetic background was used as the control. Acceptable male sterility (2.0-2.5 score) was observed in the 87DIA4 background from application at V10/0.56 lb/a glyphosate. In LH59, 4/5 events showed acceptable male sterility from V10/0.56 lb/a. For LH195 and LH198, male sterility was observed at either V10/0.56 or V12/0.56 lb/a. The corresponding treatments for NK603 inbreds were all fertile. Greenhouse evaluations of plants at the F1 stage also showed good male sterility in other backgrounds, including FBLL, LH172, LH244, and LH295.

The results obtained demonstrate that glyphosate-inducible male sterility works in multiple genotypes. The results show that the observed inducible male sterile phenotype was not due to genetic background, based on the observation of glyphosate-induced male sterility in all 4 genotypes. The results further demonstrated the ability to introduce the glyphosate-inducible male sterile phenotype into multiple genetic backgrounds.

8. Based on the identified expression profile for creation of glyphosate-inducible male-sterile plants, an analysis was carried out to identify a sampling of transformation constructs for ready generation of the herbicide-inducible male sterile phenotype. Additional constructs were prepared containing these promoters and the linked genetic elements that have demonstrated an expression profile that is high in vegetative tissue and low in male reproductive tissue and are described in Tables 1 below. Transgenic corn plants were produced that contained these constructs and were field tested for male sterility after glyphosate application. Events from each

of the constructs listed in Table 1 have demonstrated vegetative glyphosate tolerance and male sterility.

Table 1. DNA constructs that provide a male sterile phenotype in corn.

Constructs	Code	Genetic Elements
PMON58400	HAL	CaMVe35S/hsp70 intron/CP4 EPSPS-1 /nos 3'
PMON58401	HAM	CaMVe35S/hsp70 intron/CP4 EPSPS-2/nos 3'
PMON42471	HAD	CaMV35Schimeric1/hsp70 intron/CP4 EPSPS-1/nos 3'
PMON42475	HAH	CaMVe35S.ract1/ hsp70 intron/CP4 EPSPS-1/nos 3'
PMON42469	HAB	CaMVe35S/rss intron/CP4 EPSPS-1/nos 3'
PMON42476	HAI	CaMV35Schimeric2/hsp70 intron/CP4 EPSPS-1/nos 3'
PMON42474	HAG	CaMV35Schimeric3/hsp70 intron/CP4 EPSPS-1/nos 3'

9. It is my opinion, based on the studies above, the teachings in the current application, the working examples describing the creation of plants exhibiting a glyphosate-inducible male sterile phenotype, the description of the expression profile for creation of such plant, the knowledge of one of skill in the art at the time the application was filed, and my experience in agricultural biotechnology, that one of skill in the art in possession of the patent current application could readily prepare transgenic maize plants with a glyphosate-inducible male sterile phenotype using many different combinations of promoters and glyphosate resistant EPSPS transgenes without undue experimentation.

10. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Jan 15, 2013
Date

Paul C.C. Feng
Paul C.C. Feng